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RESEARCH ARTICLE

Maternal antibiotics disrupt microbiome, behavior, and temperature regulation in unexposed infant mice

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Abstract

Maternal antibiotic (ABx) exposure can significantly perturb the transfer of microbiota from mother to offspring, resulting in dysbiosis of potential relevance to neurodevelopmental disorders such as autism spectrum disorder (ASD). Studies in rodent models have found long-term neurobehavioral effects in offspring of ABx-treated dams, but ASD-relevant behavior during the early preweaning period has thus far been neglected. Here, we exposed C57BL/6J mouse dams to ABx (5 mg/ml neomycin, 1.25 μg/ml pimaricin, .075% v/v acetic acid) dissolved in drinking water from gestational day 12 through offspring postnatal day 14. A number of ASD-relevant behaviors were assayed in offspring, including ultrasonic vocalization (USV) production during maternal separation, group huddling in response to cold challenge, and olfactory-guided home orientation. In addition, we obtained measures of thermoregulatory competence in pups during and following behavioral testing. We found a number of behavioral differences in offspring of ABx-treated dams (e.g., modulation of USVs by pup weight, activity while huddling) and provide evidence that some of these behavioral effects can be related to thermoregulatory deficiencies, particularly at younger ages. Our results suggest not only that ABx can disrupt microbiomes, thermoregulation, and behavior, but that metabolic effects may confound the interpretation of behavioral differences observed after early-life ABx exposure.

KEYWORDS

dysbiosis, metabolism, microbiome, mouse model, social development, thermoregulation

1 | INTRODUCTION

The transfer of commensal microbes from mother to offspring is now recognized as a critical perinatal event for many species, including humans (Karaivazoglou et al., 2020; J. Wang & Lin, 2021). The peripartum period thus presents a distinct window of vulnerability given that administration of antibiotics (ABx) is common during this period (Bookstaver et al., 2015; Braye et al., 2018). The quality and quantity of microbiota transferred to infants can thus be disrupted by maternal ABx exposure (Ainonen et al., 2022; Diamond et al., 2021; Hill et al., 2021; Zimmermann & Curtis, 2020), with potentially wide-ranging effects for developing offspring (see Heijtz, 2016; Neuman et al., 2018; Obermajer et al., 2017; Valentine et al., 2018). In fact, early-life ABx is now thought to be a risk factor for a variety of adverse health outcomes (e.g., Hu et al., 2017; Russell et al., 2015; Timm et al., 2017; Zhong et al., 2021), including neurocognitive deficits (e.g., Slykerman et al., 2019;

Slykerman et al., 2017; but see Axelsson, et al., 2019a). Importantly, many of the somatic risks of early life ABx—for example, allergic disorders, asthma, gastrointestinal (GI) dysfunction, dysbiosis—are also common in Autism Spectrum Disorders (ASDs; e.g., Rose et al., 2018; Williams et al., 2011), suggesting that ABx exposure early in life may be relevant to the etiology and/or symptomatology of ASDs (e.g., Niehus & Lord, 2006). A growing number of epidemiological studies have added support to this hypothesis (e.g., Atladóttir et al., 2012; Axelsson, et al., 2019b; Vargason et al., 2019; but see Hamad et al., 2019; Łukasik et al., 2019).

With numerous confounding variables and potential causal pathways that may mediate associations between early-life ABx and ASDs, animal models are needed and have begun to appear (see Cusick et al., 2021; Nagpal & Cryan, 2021). Microbiome and GI abnormalities have been documented in a number of etiologically distinct rodent models of autism, including maternal immune activation via polyinosinic:polycytidylic acid (Hsiao et al., 2013; Morais et al., 2018), maternal high fat diet (Buffington et al., 2016), and prenatal exposure to the antiseizure drug valproic acid (de Theije et al., 2014a; de Theije et al., 2014b). Social differences observed in offspring in such models have even been shown, in cases, to reverse following probiotic administration (Buffington et al., 2016; Hsiao et al., 2013) or cohousing exposed mice with nondysbiotic mice (Buffington et al., 2016). There are reports of similar effects with direct manipulation of the microbiome on ASD-relevant behaviors. For example, rats and mice reared in germ-free (GF) environments—that is, in the total absence of microbiota—differ significantly from controls in tests of sociability and anxiety (e.g., Arentsen et al., 2015; Crumeyrolle-Arias et al., 2014; Desbonnet et al., 2014). Such studies include reports of a number of ASD-relevant changes in physiology and neurodevelopment, including altered HPA-axis reactivity (Crumeyrolle-Arias et al., 2014), as well as altered dopamine metabolism (Crumeyrolle-Arias et al., 2014) and levels of brain-derived neurotrophic factor (Arentsen et al., 2015) in brain.

More specific to perinatal ABx exposure, several recent studies have examined the consequences of depleting maternal microbiota with ABx for offspring behavior. For example, Tochitani et al. (2016) administered a combination of nonabsorbable ABx (i.e., ABx that are not absorbed across the gut into the rest of the body) to pregnant C57BL/6J dams from embryonic day 9 (E9) to E16 and examined offspring activity levels, anxiety-like behavior, and sociability in adulthood. Offspring of ABx-treated dams showed reduced activity levels during the dark phase as well as reduced activity and higher anxiety-like behavior in an open field as adults (Tochitani et al., 2016). Leclercq et al. (2017) administered low doses of penicillin V (phenoxymethylpenicillin) to pregnant BALB/c dams beginning a week before birth through weaning (i.e., postnatal day 21; P21), comparing offspring from these litters to the offspring of controls and dams that received ABx plus a probiotic (*Lactobacillus rhamnosus*). As adults, male offspring of ABx-treated dams displayed lower levels of anxiety on an elevated plus maze compared with males from control litters, whereas as female offspring from ABx + probiotic dams showing reduced anxiety compared with both control and ABx groups (Leclercq et al., 2017). Leclercq et al. (2017) also found reduced sociability and altered response to social novelty in offspring of ABx-treated dams in a three-chamber sociability test, as well as heightened aggressive behavior in male offspring of ABx-treated dams in a microdefeat paradigm. In addition, significant increases in both vasopressin 1B receptor (Avpr1b) and inflammatory cytokine (IL-6, IL-10, and Cxcl15) expression in the frontal cortex were found in ABx-treated mice (Leclercq et al., 2017). Several additional studies have reported changes in patterns of gene expression in brain (Champagne-Jorgensen et al., 2020; Kayyal et al., 2020; Volkova et al., 2021), changes in microglia and other aspects of neural development (Perna et al., 2021), and/or sex-dependent alterations in behavior (e.g., anxiety, sensorimotor gating, sociability; Champagne-Jorgensen et al., 2020; Kayyal et al., 2020; Perna et al., 2021) in offspring following exposure of dams to ABx.

Such studies provide clear evidence that ABx can induce long-term ASD-relevant neurobehavioral changes in rodent models. ASDs are nonetheless developmental disorders, with many core symptoms detectable during the first years of life (e.g., Bacon et al., 2017; Bedford et al., 2017). Thus, the current work focuses on markers of early social and communicative development in offspring of ABx-treated compared with the offspring of non-ABx-treated dams in C57BL/6J mice. This report stems from a larger project investigating the effects of perinatal ABx on social-emotional development across the lifespan, including analyses of interactions between mothers and offspring early in life. Here, we report an initial examination of offspring social behavior, including ultrasonic vocalization (USV) in response to maternal separation, huddling in response to cold challenge, and attraction to home cage odors during early development. These assays represent an ethologically grounded battery designed to capture a range of social and emotional behaviors, as well as basic regulatory capabilities in developing mice. We expected that perinatal exposure of dams to ABx would significantly alter the microbiota of the offspring. Even without direct exposure to the ABx, affected offspring were predicted to show behavioral and physiological changes, including disruptions in their early social and emotional development and concomitant changes in homeostatic regulation.

2 | MATERIALS AND METHODS

All animal care and experimental procedures were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) at Indiana University (IU #15-027) and conducted in accordance with both the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and international standards. Care was taken at every step to minimize pain and discomfort to animals.

2.1 | ABx treatment model and experimental subjects

2.1.1 | ABx exposure

All male and female breeders were C57BL/6J purchased from Jackson Labs (Sacramento, CA). Mice were housed in standard cages

(30 × 13 × 19 cm) with food and water available ad libitum, in a vivarium maintained at 22 ± 2°C on a 14:10 h light/dark cycle (lights on at 0800 h). Females were group housed until pairing with males at ~8–12 weeks of age. Females were inspected daily and paired with males only when there were visible signs of proestrus or estrus (Byers et al., 2012). Following pairing, females were checked daily for the presence of a vaginal plug. If a plug was found, the female was separated and placed in her own cage. Otherwise, females remained with males and all females were separated after 3–4 days of being paired. The day of vaginal plug detection or transfer from the male cage was considered gestational day (G) 0 for each litter. On G12, females determined to be pregnant based on weight gain were assigned to either the ABx-treated or control condition. The day of birth was designated P0.

Dams in the ABx condition received nonabsorbable ABx dissolved in water, administered via voluntary drinking from G12 to P14. ABx administration thus began soon after confirmation of pregnancy and ended prior to pups emerging from the nest, when they begin sampling water from water bottles. We started with the ABx recipe reported by Tochitani et al. (2016), in which a combination of bacitracin, neomycin, and pimaricin were administered to pregnant C57BL/6J dams in drinking water and reported to significantly alter the maternal gut microbiome. Nevertheless, during piloting, we found that dams rejected water containing various levels of bacitracin, including the 5 mg/ml dose reported by Tochitani et al. (2016). We thus excluded bacitracin and our final ABx "cocktail" consisted of 5 mg/ml neomycin (Sigma–Aldrich, St. Louis, MO, USA), 1.25 μg/ml pimaricin (Sigma–Aldrich; dissolved in acetic acid, 5 mg/ml), and .075% (v/v) acetic acid dissolved in drinking water (cf. Tochitani et al., 2016). ABx solution was refrigerated and remixed every 10 days. Apart from the presence or absence of ABx in their drinking water, dams were treated identically. Water was provided through the cage lid via a modified graduated cylinder with a rubber stopper and stainless-steel drinking spout (Bachmanov et al., 2002). Quantity consumed was recorded daily and water replenished when needed. All dams were weighed on G0, G3, or G4 (collapsed for analysis) and G7 through the day of birth (G18 of G19) and at least once every 3 days until P21 for dams with litters or the equivalent of G40 for females that were either not pregnant or did not give birth to a surviving litter.

2.1.2 | Experimental subjects

A total of 72 females ($N = 32$ ABx) were used for the analysis of the effects of ABx on dam weight and water consumption, as well as litter outcomes. Three additional litters were excluded due to abnormally long gestation ($N = 1$) or gaps in record keeping ($N = 2$). Only litters with (1) G0 confirmed by vaginal plug, (2) subsequently born by G19, and (3) consisting of at least two male and two female pups were used in the study. A total of 84 offspring ($N = 44$ from ABx-treated dams and $N = 40$ from control dams) derived from 21 litters served as subjects. Litters were culled to $N = 4$ pups per litter (two males, two females) on P2 and each pup was tattooed for individual identification via a single needle-prick to the underside of one paw.

2.2 | Experimental design

The full design of the study is illustrated in Figure 1. Briefly, experimental dams voluntarily consumed ABx-containing water from G12 to P14. Feces from dams and pups were collected on G12 (prior to ABx administration), P14 (dams), and P21 (dams and pups). Fecal samples were stored on dry ice during sampling and at −80°C thereafter until assay. Behavioral tests were employed to assay the normative social behavior displayed by young, preweanling (P7–P12) mice. This included tests of maternal separation (P7 and P9), huddling (P8 and P10), and homecage odor preference (P7, P9, and P12). At weaning (P21), pups and dams were perfused to collect blood and brains, which were stored at −80°C for later analysis. Data from these samples are not reported here. Pup weight and tail length were also measured repeatedly across development, the latter because of its known importance for thermoregulation (Barnett, 1965; Rand et al., 1965).

2.3 | Gut microbiome analysis

Although fecal samples were obtained from the dams and offspring that contributed to the behavioral outcomes in the present report, some of these were lost due to difficulties with a commercial kit. We also subsequently refined our methods for sample collection and storage. DNA extraction, 16S sequencing, and analyses of fecal microbiota were thus performed on samples obtained from subsequent cohorts of animals, exposed to an identical regime of perinatal ABx. Full exposition of these results is beyond the scope of the current paper, but to validate the effectiveness of our ABx treatment regime for altering the gut microbiome we present here an analysis of alpha-diversity (within sample diversity, quantified using the Simpson index). To this end, we analyzed fecal samples from 16 dams (eight ABx, eight control) measured at three time points relative to offspring development: gestational day 12 (G12—baseline pretreatment), P14 (end of ABx exposure), and P21 (weaning). Fecal samples from two male and two female offspring from each litter at P21 were also analyzed.

DNA was extracted from dams' and pups' feces using a commercial DNA extraction kit (Promega Maxwell RSC Tissue DNA Kit, Madison, WI). To prepare samples for DNA extraction, each sample was homogenized in 80 μl TE buffer, 20 μl RNA A solution, and 250 μl lysis buffer transferred from a well in each cartridge of the kit and centrifuged at 4°C for 5 min at 1200 rpm, and then its supernatant was transferred back to the well for the extraction. Verification of the quality of extracted DNA and sequencing for bacterial DNA were conducted at the IU Center for Genomics and Bioinformatics. The quantity and purity of extracted DNA were verified using Agilent TapeStation DNA analysis (Santa Clara, CA), and multiplexed amplicon libraries for V4 domain of microbial 16S ribosomal RNA (rRNA) genes were prepared using NECTflex 16S V4 Amplicon-Seq Library Prep Kit 2.0 (Bioo Scientific, Austin, TX). 16S gene sequencing reads were analyzed via Divisive Amplicon Denoising Algorithm 2 (DADA2; Callahan et al., 2016) by the IU Center for Genomics and Bioinformatics at Indiana University. R 3.4.3 was used for alpha-diversity. Briefly, for the alpha diversity

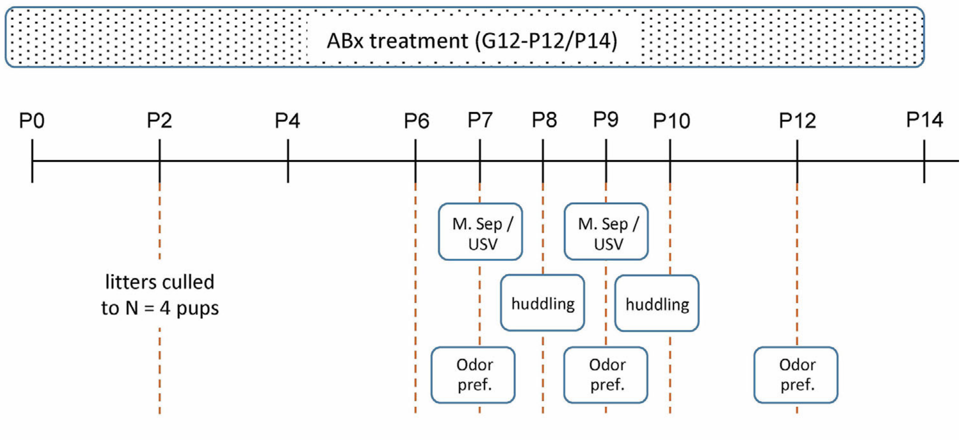

FIGURE 1 Study design. Antibiotics (ABx) were administered to dams via drinking water from gestational day 12 through offspring postnatal (P) day 14 (P14). Litters were culled on P2. Tests of maternal separation were performed on P7 and P9. Homecage odor preference tests were administered on P7, P9, and P12, and huddling tests were administered on P8 and P10. Fecal samples were collected from dams and pups on G12, prior to the first administration of ABx, P14 (dams), and P21 (dams and pups)

analysis, the two samples with less than 10,000 reads post-DADA2 were excluded (one from a male pup, and one from a dam at P14). The replicon sequence variant table was then rarefied to a depth of 10,586 reads per sample. Simpson Index for all remaining samples was calculated in R using the vegan package (Oksanen et al., 2020).

2.4 | Behavioral tests

2.4.1 | Maternal separation/USV (P7 and P9)

Pups were tested on P7 and P9 during episodes of maternal separation, using a modified version of Hofer et al. (2002) protocol. Prior to testing, the dam was removed and placed in a clean cage with ad libitum food and water. The homecage containing the pups was then moved to a warm incubator set to 32°C. Next, pups were individually placed on a nonconductive platform in a temperature-controlled chamber. Ambient temperature (T_a) in the chamber was set to 23°C (mean = 22.7 ± .05°C) and did not differ between conditions at either P7 ($t_{19} = .42$, $d = .18$, $p = .68$) or P9 ($t_{17} = .75$, $d = .34$, $p = .47$).[1] The test duration was 5 min, during which USVs were measured using a Pettersson D240X ultrasound detector (Pettersson Elektronik AB, Uppsala, Sweden) set to 50–60 kHz, positioned at the upper edge of the chamber. Simultaneously, infrared thermographs were obtained once per min, using an ICI 9640-P infrared camera (Infrared Cameras Inc.; ICI; Beaumont, TX) and IR Flash ver. 2.0 (ICI). At the end of each test, the pup was immediately removed from the chamber and its rectal temperature (T_{rectal}) measured, using an RET-4 (.032″ dia) temperature probe for neonatal mice, inserted for 15 s. Tail length and body weight were also measured prior to the mouse being returned to the warm incubator. The chamber was then cleaned using a Roccal-D solution and allowed to dry prior to the next test. Gloves were worn during transfer and handling of pups to minimize heat flux (Blumberg et al., 1992b).

2.4.2 | Huddling/group regulation (P8 and P10)

Huddling tests involved simultaneous testing of all pups in the litter on P8 and P10. All such tests were performed on a circular platform (dia = 11.25 cm) within a double-walled glass chamber (height = 30 cm; dia = 15.2 cm; see Harshaw & Alberts, 2012; Harshaw et al., 2014). Ambient temperature (T_a) was controlled by circulating temperature-controlled water through the chamber walls and was monitored and logged using a Type K thermocouple located 1.5 cm above the platform, connected to an Omega HH802U thermometer and Omega Software for Windows, Ver. 1.6 (Omega Engineering, Inc., Stamford, CT). An ICI 9640-P infrared camera (ICI) and video camera were mounted above the chamber, such that both could simultaneously image pups at angles nearly perpendicular to the testing platform, once per min. Thermographs were acquired using IR Flash ver. 2.0 (ICI); video frame grabs were acquired using Scion Image 1.62a. Because fur development is extensive by P10, infrared thermographs were obtained and analyzed only for P8 pups.

Pups were removed from their dams, weighed, and each pup marked with a nontoxic water-based paint for individual identification. All pups were then placed in the testing chamber with T_a stabilized at ~33.0°C (mean: 33.1 ± .05°C). To standardize initial conditions, a Plexiglas divider created a separate compartment for each pup, preventing contact. Once in the chamber, pups were provided 10 min to acclimate and regain heat lost during transfer (Blumberg et al., 1992a). The divider was then removed and data collection initiated. Testing involved a standardized sequence consisting of a warm and a cool phase. During the warm phase, the initial warm T_a was maintained for a further 51 min. Water baths were then switched and T_a cooled rapidly to ~21.5°C (mean: 21.7 ± .07°C). The cool phase consisted of a further 51 min

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[1] Temperature records were unavailable for two of the P9 ABx runs due to equipment failure and/or experimenter error.

of data collection, initiated once T_a reached ~23°C. Trials thus lasted ~120 min. At the end of the trial, each pup was removed from the chamber, its T_{rectal} and tail length measured, and then returned to its dam. Importantly, T_a did not differ between conditions during either the warm or cool phases at P8 ($t_{18} = .47$, d = .22, $p = .65$ and $t_{17} = 1.85$, d = .77, $p = .08$, respectively) or P10 ($t_{19} = .69$, $d = .31$, $p = .50$ and $t_{19} = .48$, $d = .21$, $p = .63$, respectively).[2]

Huddling tests permit the calculation of both aggregative metrics of pups as a litter as well as measures of individual pups in the litter. To assess individual differences in locomotor activity we obtained pup location and calculated movement from video frames, sampled once per minute, using a custom NIH ImageJ script (Harshaw & Alberts, 2012). The tip of each pup's nose and the base of its tail were first identified (or estimated, when obscured) and marked for each frame. The midpoint of a line between the *x,y* coordinates for these points was then calculated, such that pup activity (i.e., movement of a pup's midpoint, in cm/min) could be calculated across consecutive frames. Pups are generally quiescent and inactive during the warm phase, so activity was scored only for the cool phase of each test. The cohesiveness of huddles was quantified by counting the number of discontinuous clumps of pups in each frame, excluding contacts made merely from tails or outstretched paws (see Harshaw & Alberts, 2012). For example, if all four pups were separate a score of "4" was assigned, whereas if all pups formed a single clump a score of "1" was assigned. Infrared thermographs for the cool phase of the huddling test were analyzed using custom Matlab code. We extracted and averaged the warmest 10% of pixels, to provide a measure of huddle temperature (T_{huddle}).

2.4.3 | Home cage odor preference (P7, P9, and P12)

Pups were given a 3 min test of homecage odor preference three times, on P7, P9, and P12, using a variant of Meyer and Alberts' (2016) procedure. To avoid variation due to eye opening, all tests were conducted in darkness. The apparatus consisted of a rectangular Plexiglas chamber (4.5″ × 7.5″ × 3″ high) with plastic mesh flooring, divided into two 1.5″ approach zones, located on either end of the chamber and a 4.5″ neutral zone in the center. Odor fields were created by placing 4.5″ × 1.5″ acrylic trays in compartments beneath the floor of the chamber, each containing 7 g of bedding gathered from either the home cage of the test animals or an age-matched litter. Pups were removed from their dam and placed, as a group, in a plastic holding container for ~30 min prior to being tested one at a time (see Meyer & Alberts, 2016). For each test, the pup was placed in the center of the apparatus and observed via a video monitor in an adjacent room. All tests were conducted at room temperature (mean = 20.4 ± .08°C). During these tests an experimenter observed and scored the pup as entering an approach zone whenever both ears crossed the boundary between that zone and the center (i.e., neutral) zone, using a custom Visual Basic/Excel macro. Between each test, the chamber was cleaned with 70% ethanol solution and the position of the odor trays counterbalanced.

2.5 | Statistical analysis

All analyses were performed in *R*, version 4.1.0 (R Core Team, 2021), using a 5% criterion for significance (two-tailed). Outlier detection was performed using Grubbs' test, as implemented in the "outliers" package (Komsta, 2015). Given the hierarchical design, data were analyzed primarily via linear mixed effects (LME) models constructed using the *lmer* function from the "lme4" package via the restricted maximum likelihood approach (Bates et al., 2015). When tests were administered or measures taken multiple times (e.g., maternal separation, weight, water consumption), models were constructed using a repeated-measures approach, with individuals nested within litters and litters nested within breeding groups (or individual dams nested within breeding groups, where appropriate). When variables such as pup weight or rectal temperature (T_{rectal}) were included as covariates these were normalized (i.e., converted to *Z* scores), within sex. *F*-statistics, *p* values, and degrees of freedom were obtained via Satterthwaite's approximation, using the "lmerTest" package (Kuznetsova et al., 2016). Effect sizes for LME models are partial ω^2—an extension of R^2 (see Xu , 2003)—calculated using the "effectsize" library (Ben-Shachar et al., 2020). Effect sizes reported for post-hoc tests were calculated using the "emmeans" library (Lenth, 2020).

3 | RESULTS

3.1 | Maternal ABx treatment depletes gut microbiota of dams as well as that of their unexposed offspring

As illustrated in Figure 2, exposure of dams to ABx resulted in significant shifts in the alpha diversity of fecal microbiota in both dams and offspring. A repeated measures LME model controlling for breeding group and dam as nested random factors revealed significant main effects of time ($F_{2,27.6} = 120.6$, $\omega^2 = .89$, $p < .00001$), ABx treatment ($F_{1,14.2} = 192.0$, $\omega^2 = .92$, $p < .00001$), and time × treatment interaction ($F_{2,27.6} = 119.2$, $\omega^2 = .89$, $p < .00001$; see Table S1). Specifically, alpha diversity was significantly lower in ABx-treated dams at P14 ($t_{35.5} = 16.8$, E.S. = 10.48, $p < .00001$) and P21 ($t_{34.4} = 13.6$, E.S. = 8.24, $p < .00001$) but did not differ from control dams prior to ABx treatment (i.e., G12; $t_{34.4} = .02$, E.S. = −.01, $p = .98$). A LME model for alpha diversity in pup fecal samples revealed main effects of sex ($F_{1,45.3} = 9.85$, $\omega^2 = .16$, $p < .003$) and ABx treatment ($F_{1,14.1} = 13.0$, $\omega^2 = .42$, $p < .003$), as well as significant sex × ABx treatment interaction ($F_{1,45.3} = 11.5$, $\omega^2 = .18$, $p < .002$; see Table S2). As illustrated in Figure 2, offspring of ABx dams had significantly lower alpha diversity than offspring of control dams ($t_{14.1} = 3.61$, E.S. = 1.76, $p < .003$), an effect that was more pronounced in males ($t_{22.4} = 4.74$, E.S. = 2.62, $p < .0001$) than females ($t_{21.8} = 1.65$, E.S. = .91, $p = .11$). These results confirm

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[2] Temperature logs were unavailable for three P8 control runs (once during the warm phase, twice during the cool phase) due to equipment failure and/or experimenter error.

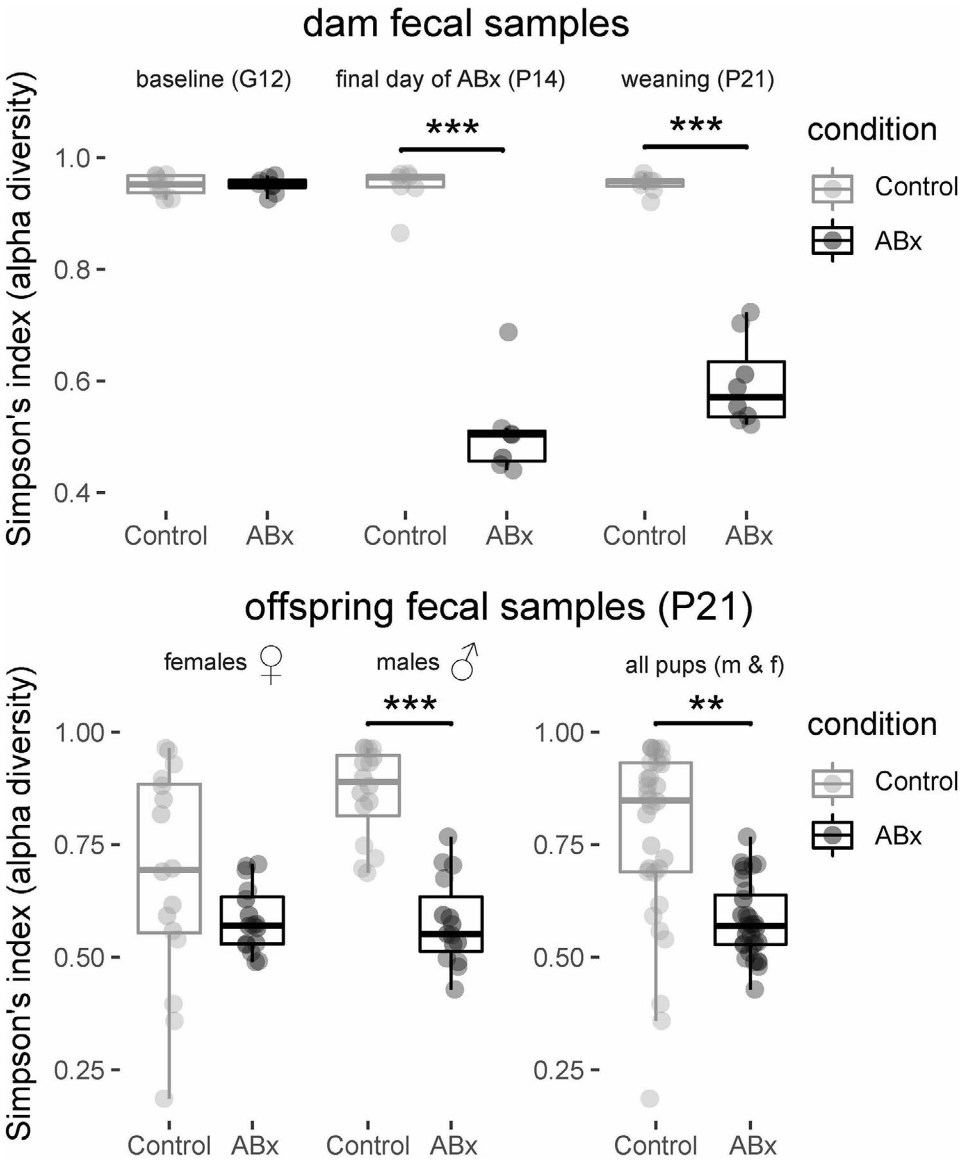

FIGURE 2 Simpson's index, a measure of alpha diversity or microbial richness of fecal samples obtained from dams at offspring age G12 (baseline, pre-ABx), P14 (final day of ABx), and P21 (weaning). Data are depicted as boxplots with overlaid, jittered data points. The upper panel depicts Simpson's index for fecal samples obtained from dams, whereas the lower panel depicts the same measure for unexposed offspring (two males and females from each litter) at P21. Note that the samples depicted were obtained from different cohorts of mice than those contributing to the behavioral and physiological outcomes reported in the present paper (see *Materials and Method*). A double asterisk (**) denotes significance at $p < .01$. Triple asterisks (***) denote significance at $p < .001$

that our ABx regime alters the transfer of microbiota from dam to offspring.

3.2 | ABx did not alter dam weight, water intake, or fecundity

Exposure of dams to ABx had no effects on overt indicators of maternal health, litter makeup, or pup survival. Weight did not differ between conditions at baseline (i.e., G0) in either pregnant (ABx: 20.3 ± .26 g; Con: 20.2 ± .23 g; $t_{61} = .15$, $d = .37$, $p = .71$) or nonpregnant (ABx: 18.0 ± .85 g; Con: 18.8 ± .31 g; $t_7 = 1.04$, $d = .76$, $p = .33$) females (see Figure S1). A LME model for dam weight revealed significant main effects of time (before, during, or after ABx exposure, from G12-P14; $F_{2,135} = 57.6$, $\omega^2 = .45$, $p < .00001$) and pregnancy ($F_{1,66.5} = 69.7$, $\omega^2 = .50$, $p < .00001$) as well as significant interaction between time and pregnancy ($F_{2,135} = 48.7$, $\omega^2 = .40$, $p < .00001$) but no effect of ABx treatment ($F_{1,58.1} = .03$, $\omega^2 = -.02$, $p = .87$; see Table S3). A model restricted to mothers of litters used to examine pup outcomes in the present report similarly showed a main effect of time ($F_{2,38} = 198.1$, $\omega^2 = .91$, $p < .00001$) but not ABx treatment ($F_{1,15.9} = .36$, $\omega^2 = -.04$, $p = .56$) on dam weight (see Table S4).

To verify ABx exposures and to ensure that ABx-treated dams were not dehydrated, water consumption was analyzed (see Figure S2). An

LME model revealed significant main effects of time (before, during, or after G12–P14; $F_{2,135.2}$ = 94.8, ω^2 = .58, p < .00001) and pregnancy ($F_{1,67.9}$ = 34.0, ω^2 = .32, p < .00001) as well as time x pregnancy interaction ($F_{1,66.4}$ = 69.6, ω^2 = .23, p < .00001) but no effect of ABx treatment ($F_{1,67.9}$ = .74, ω^2 = .00, p = .39) on water consumption (see Table S5). A model restricted to only mothers of litters used in the present study similarly showed a main effect of time ($F_{2,38}$ = 249.4, ω^2 = .92, p < .00001) but not ABx treatment ($F_{1,19}$ = .03, ω^2 = –.05, p = .88; see Table S6).

3.3 | Effects of ABx on litter composition and survival

ABx exposure did not alter the probability that dams gave birth to a live litter (ABx: 30 out of 32; Con: 33 out of 40; χ^2 = 1.16, w = 1.02, p = .28) or litter survival (ABx: 27 out of 30; Con: 25 out of 33; χ^2 = 1.33, w = 1.02, p = .25). Litter size at birth also did not differ between treatment conditions (ABx: 5.63 ± .35; Con: 5.56 ± .33; t_{50} = .15, d = .04, p = .89). Sex ratio of litters was similarly unaffected by ABx treatment (ABx: .53 ± .05 male; Con: .48 ± .04 male; t_{50} = .72, d = .2, p = .48).

3.4 | Physical development of offspring appears unaffected by maternal ABx

There was no significant difference in pup weight at P2 (ABx: 1.50 ± .02 g; Con: 1.45 ± .03 g; $t_{67.9}$ = 1.33, d = .30, p = .19; see Figure S3). A model controlling for litter and pup as nested random factors revealed significant effects of age ($F_{6,465.8}$ = 2223, ω^2 = .98, p < .00001) and sex ($F_{1,61.4}$ = 8.77, ω^2 = .11, p < .005) but not dam ABx treatment ($F_{1,19}$ = .95, ω^2 = .00, p = .34) on pup weight. A model for tail length similarly showed an effect of pup age ($F_{3,224.1}$ = 3086.3, ω^2 = .97, p < .00001) but not dam ABx treatment ($F_{1,18.9}$ = .73, ω^2 = –.01, p = .4).

3.5 | Maternal separation test

3.5.1 | USVs differ between sexes in controls but not in ABx offspring and differ in relation to body weight

A LME model for pup USVs[3] showed significant main effects of sex ($F_{1,57.7}$ = 8.49, ω^2 = .11, p < .01) and age ($F_{1,74.6}$ = 15.2, ω^2 = .16, p < .0005) but not ABx treatment ($F_{1,18.2}$ = .08, ω^2 = –.05, p = .79). Female pups vocalized more than males ($t_{57.7}$ = 2.89, E.S. = .7, p < .006) and pups vocalized more on P9 than on P7 ($t_{74.5}$ = 3.92, E.S. = .63, p < .0003). In addition, significant weight × condition ($F_{1,68.6}$ = 7.04, ω^2 = .08, p < .01) and weight × age × condition ($F_{1,75.5}$ = 4.23, ω^2 = .04, p < .05) interactions were found (see Table S7).

Figures 3 and S4 depict weight × condition interaction for USVs. A significant negative correlation between pup weight and USV production was found for offspring of ABx-treated dams (P7: t_{42} = 1.42, r = –.21, p = .103; P9: t_{42} = 4.13, r = .54, p < .0002; P7/P9 avg: t_{42} = 3.5, r = –.47, p < .002) that was absent or slightly positive for offspring of control dams (P7: t_{34} = .64, r = .11, p = .53; P7: t_{36} = 1.49, r = .13, p = .24; P7/P9 avg: t_{36} = 1.66, r = .27, p = .11). That is, lighter pups from ABx litters tended to emit more USVs, whereas lighter pups from control litters tended to emit fewer USVs. As can be seen in the lower panel of Figure 3, however, the modulation of USVs by weight in pups from ABx litters was significant at P9 (t_{42} = 4.13, r^2 = .29, p < .0002) but not P7 (t_{42} = 1.42, r^2 = .05, p = .16).

3.5.2 | Thermoregulation by pups during maternal separation: ABx pups cool faster

A model for rectal temperature (T_{rectal}) after 5 min of maternal separation revealed significant effects of pup weight ($F_{1,124.8}$ = 8.08, ω^2 = .05, p < .01) and age ($F_{1,128.9}$ = 56.28, ω^2 = .3, p < .00001) as well as significant weight × age ($F_{1,129.4}$ = 4.43, p < .05), age × condition ($F_{1,128.9}$ = 4.55, ω^2 = .03, p < .05), and weight × sex × condition ($F_{1,136.3}$ = 4.06, ω^2 = .02, p < .05) interactions (see Table S8). Pups had significantly higher T_{rectal} values on P9 than P7 pups ($t_{128.9}$ = 7.5, E.S. = 1.19, p < .00001) and pups from ABx-treated dams tended to be cooler than pups from control dams on P7 (27.7 ± .17°C vs. 28.2 ± .16°C; $t_{18.5}$ = 1.80, E.S. = .61, p = .088) but not P9 (28.5 ± .14°C vs. 28.7 ± .14°C; $t_{19.5}$ = .21, E.S. = .07, p = .84).

An analysis of infrared measures of skin surface temperature revealed a similar and even clearer pattern. In particular, a model for interscapular temperature (T_{IS}) averaged over the last two min of testing ($T_{\text{IS}}^{\text{end}}$) showed significant effects of pup weight ($F_{1,147}$ = 6.43, ω^2 = .04, p < .02) age ($F_{1,138.7}$ = 63.52, ω^2 = .31, p < .00001) and condition ($F_{1,143}$ = 7.01, ω^2 = .04, p < .01), as well as sex × condition ($F_{1,138.7}$ = 4.1, ω^2 = .02, p < .05), age × condition ($F_{1,138.7}$ = 4.24, ω^2 = .02, p < .05) and sex × age × condition ($F_{1,138.6}$ = 5.38, ω^2 = .03, p < .025) interactions (see Table S9). As illustrated in Figure 4, pups from ABx litters had significantly cooler interscapular regions than pups from control litters at the end of separation on P7 (31.9 ± .08°C vs. 32.2 ± .07°C; t_{147} = 3.41, E.S. = .84, p < .0001) but not P9 (32.5 ± .06°C versus 32.6 ± .07°C; t_{147} = .73, E.S. = .19, p = .47). Critically, baseline T_{IS} (i.e., average during the first 2 min of test) did not differ for pups from ABx compared with control litters at either P7 (33.8 ± .11°C vs. 33.7 ± .12°C; $t_{1,80}$ = .26, d = .06, p = .80) or P9 (33.6 ± .11°C vs. 33.6 ± .14°C; $t_{1,80}$ = .30, d = .07, p = .76), suggesting that pups of ABx-treated dams cooled more rapidly than controls as a result of the temperature challenge engendered by 5 min separation on P7. Figure S5 (upper right) illustrates sex × condition interaction, in which female offspring of ABx-treated dams were significantly cooler than control females (t_{147} = 3.27, E.S. = .32, p < .002) but there was no effect of treatment in males (t_{147} = .78, E.S. = .19, p = .44). As can be seen in

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[3] Two subjects from a single control litter (one male, one female) were significant outliers on rectal and/or infrared measures of temperature regulation and were thus removed from analyses for the maternal separation test (Grubbs tests, all ps < .05).

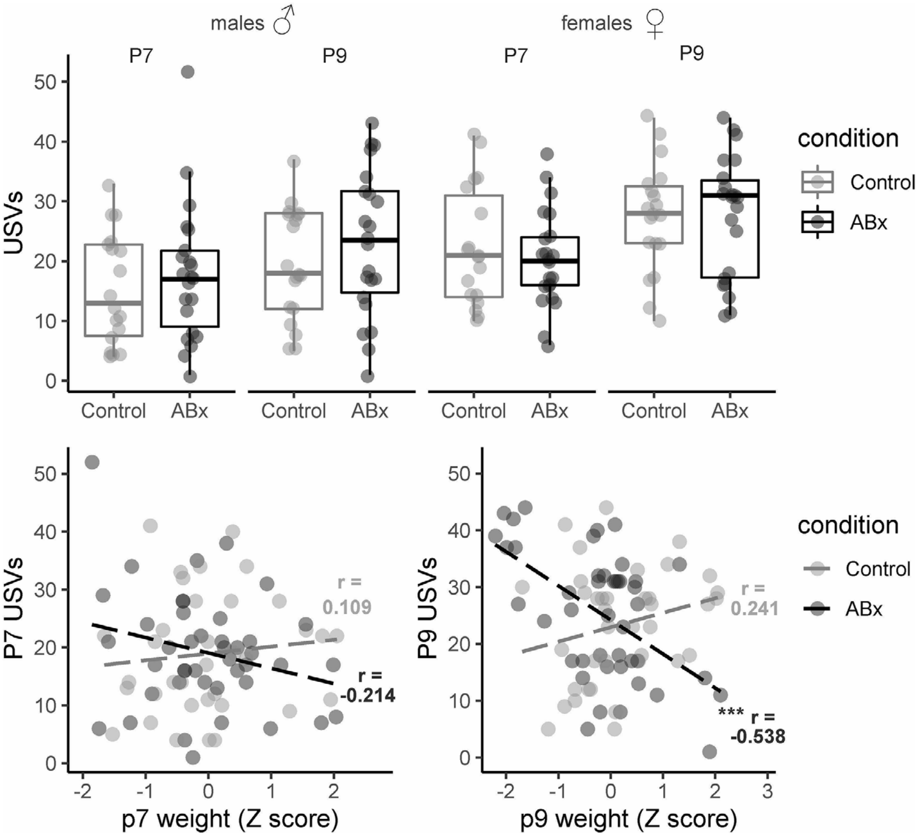

FIGURE 3 Upper panel: Boxplots illustrating ultrasonic vocalization (USV) production (i.e., number of 5 s bins in which USVs occurred) during 5 min maternal separation by sex, age (i.e., P7 or P9), and condition (i.e., offspring of ABx-treated vs. control dams). Lower panels: Relationship between USV production and offspring weight by age and condition. Correlations (*r* values) shown are Pearson product moment correlations. A double asterisk (**) denotes significance at $p < .001$

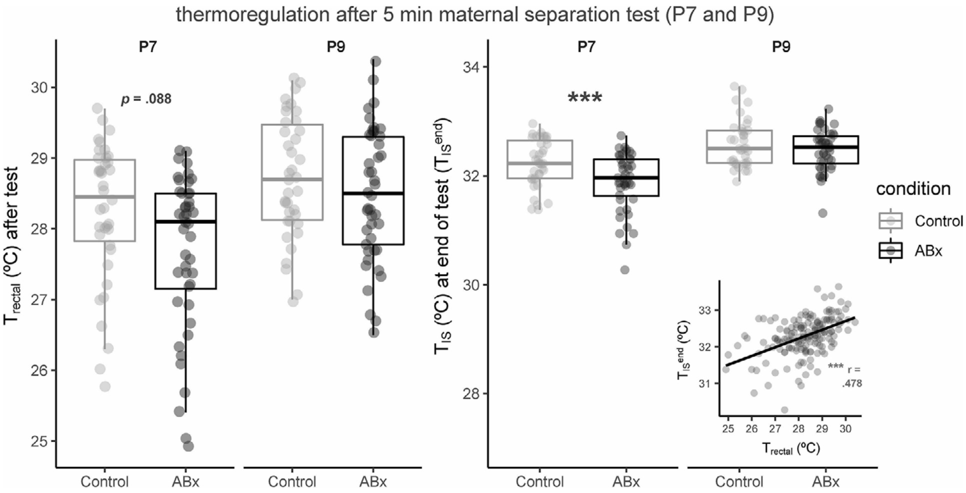

FIGURE 4 Measures of thermoregulation obtained after 5 min maternal separation test, as a function of dam treatment condition (i.e., offspring of ABx-treated versus control dams) at P7 and P9. Data are displayed as boxplots with overlaid, jittered data points. The left panel displays rectal temperature (T_{rectal}) taken immediately after the test. The right panel displays the average temperature of the interscapular region (T_{IS}) during the last two min of testing (i.e., $T_{\text{IS}}^{\text{end}}$) quantified via infrared thermography. A single asterisk (*) denotes significance at $p < .05$. Triple asterisks (***) denote significance at $p < .001$. The inset figure in the lower right-hand corner displays a scatterplot of T_{rectal} and $T_{\text{IS}}^{\text{end}}$ with a regression line from a linear fit of the data. *R*-value shown is for a Pearson product–moment correlation. As can be seen in the inset, there was a highly significant correlation between T_{rectal} and $T_{\text{IS}}^{\text{end}}$ ($t_{161} = 6.91, p < .00001$)

the lower panel of Figure S5, the former effect was driven by the cooler $T_{\text{IS}}{}^{\text{end}}$ values of female offspring of ABx-treated compared with female offspring of control dams on P7 ($t_{145.2} = 4.59$, E.S. $= 1.54$, $p < .0001$).

3.5.3 | Interaction between body temperature and USV production

To address whether difference in temperature regulation affected USV production, T_{rectal} × age × condition interaction was added to the original model for USVs. This addition not only improved model fit ($\chi^2 = 10.0$, $p < .04$) but shifted the pattern of significance. The main effects of sex ($F_{1,57.6} = 8.98$, $\omega^2 = .12$, $p < .005$) and age ($F_{1,73.8} = 9.95$, $\omega^2 = .11$, $p < .005$), as well as weight × condition ($F_{1,62.1} = 9.2$, $\omega^2 = .11$, $p < .005$) interaction were unchanged; however, significant condition × T_{rectal} interaction emerged ($F_{1,53.1} = 5.41$, $\omega^2 = .07$, $p < .025$; see Table S10). This interaction is illustrated in Figure S6, which depicts a significant negative correlation between T_{rectal} and USVs for controls ($t_{1,75} = 2.72$, $r = -.31$, $p < .008$)—that is, cooler pups vocalizing more and warmer pups less—that was absent for offspring of ABx dams ($t_{1,42} = .12$, $r = -.02$, $p = .90$). The addition of $T_{\text{IS}}{}^{\text{end}}$ to the model for USVs, in contrast, produced results largely similar to the original model (see Table S11). Taken together, these analyses suggest that the increase in USV production in pups of ABx-treated dams (see Figure 3) was likely driven by thermoregulatory differences in these pups (Figure 4). The weight × condition interaction, which was significant in all models, in turn, indicates that maternal ABx exposure altered the modulation of USV production by pup weight (see Figure 3).

3.6 | Huddling behavior

3.6.1 | Reduced activity of pups within a huddle during cool phase of temperature cycle

A repeated measures LME model across P8 and P10 for pup activity during the cool phase revealed a significant main effect of age ($F_{1,140} = 37.4$, $\omega^2 = .204$, $p < .00001$) and age × condition interaction ($F_{1,140} = 10.7$, $\omega^2 = .06$, $p < .005$). Younger pups were more active than older pups ($t_{140} = 6.12$, E.S. $= .94$, $p < .0001$; see Table S12) and, as can be seen in the left panel of Figure 5, pups from ABx-treated dams were less active than pups from control litters when tested on P8 ($t_{22.8} = 2.58$, E.S. $= .71$, $p < .02$) but not P10 ($t_{23.2} = 1.1$, E.S. $= .3$, $p = .28$).[4]

3.6.2 | Huddle cohesiveness was not affected by maternal ABx

A repeated measures LME model for cohesiveness revealed significant mains effect of temperature phase (warm vs. cool; $F_{1,67.9} = 246.3$, $\omega^2 = .78$, $p < .00001$) and age ($F_{1,67.9} = 2.14$, $\omega^2 = .05$, $p < .05$) but no effect of ABx treatment ($F_{1,62.6} = .24$, $\omega^2 = -.01$, $p = .63$; see Table S14). Huddle cohesiveness was higher during the cool phase ($t_{67.9} = 15.7$, E.S. $= 3.43$, $p < .00001$; see Figure 5) and P10 huddles were more cohesive than P8 huddles ($t_{67.9} = 2.66$, E.S. $= -.47$, $p < .02$).

3.6.3 | Temperature regulation in response to cold challenge

A repeated measures LME model for T_{rectal} at the end of testing on P8 and P10 revealed an effect of weight ($F_{1,157.9} = 4.22$, $\omega^2 = .02$, $p < .05$) and a significant age × ABx treatment interaction ($F_{1,140.1} = 24.4$, $\omega^2 = .14$, $p < .00001$; see Table S15). Pups from ABx litters were cooler than pups from control litters on P8 ($t_{20.2} = 3.04$, E.S. $= 1.37$, $p < .007$) but not P10 ($t_{20.3} = .34$, E.S. $= .15$, $p = .88$; left panel of Figure 6). This difference was corroborated using a measure of whole-litter temperature from thermographs obtained on P8. A repeated measures LME model examining the average temperature of the top 10% of pixels (T_{huddle}) at the beginning versus end of the cool phase revealed significant main effects of timing ($F_{1,16} = 14.7$, $\omega^2 = .43$, $p < .005$) and ABx treatment ($F_{1,13.4} = 7.6$, $\omega^2 = .3$, $p < .02$), as well as several interactions, including timing × condition ($F_{1,16} = 6.78$, $\omega^2 = .24$, $p < .02$; see Table S16). As can be seen in the right panel of Figure 6, T_{huddle} fell significantly during the cool phase ($t_{16} = 7.76$, E.S. $= 2.53$, $p < .00001$) and litters of pup from ABx dams were cooler than control litters at the end ($t_{19.5} = 2.28$, E.S. $= 1.74$, $p < .05$) but not beginning ($t_{24.4} = .26$, E.S. $= .43$, $p = .77$) of this period.

3.7 | Home cage odor preference

First, we examined pup activity levels during the test (i.e., total number of entries to the two odor fields). A repeated-measures LME model showed a main effect only of pup age ($F_{2,219.9} = 39.3$, $\omega^2 = .26$, $p < .00001$; see Table S17), with activity levels gradually increasing from P7 to P12 ($ps < .05$). Consistent with this, a model for latency to approach the homecage odor side revealed a main effect only of pup age ($F_{2,148} = 9.68$, $\omega^2 = .10$, $p < .0005$)—with latency to approach the homecage odor decreasing across P7–P12 ($ps < .05$)—but no significant effect of treatment condition ($F_{1,66.8} = .00$, $\omega^2 = -.02$, $p = .97$; see Table S18 for full results).

In contrast, an LME model for the proportion of total duration of time (PTD) spent on the home odor side relative to the total amount of time spent on both sides revealed a significant main effect of condition ($F_{1,77.9} = 3.97$, $\omega^2 = .036$, $p < .05$; see Table S19). Pups from litters of ABx-treated dams spent more time, overall, on the homecage odor side than offspring of control dams ($t_{77.9} = -1.99$, E.S. $= -.32$, $p < .05$; left panel of Figure 7). As illustrated in the right panel of Figure 7, this effect was driven primarily by older pups. In particular, pups from ABx-treated dams showed a greater preference for homecage odors than did pups from control litters on P12 ($t_{206.4} = 2.53$, E.S. $= .60$, $p < .02$) but not P7 ($t_{212.5} = .58$, E.S. $= -.15$, $p = .56$) or P9 ($t_{207.3} = .85$, E.S. $= -.21$, $p = .40$).

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[4] To verify that this was not driven by slight differences in T_a, we constructed a second model that included T_a and thus excluded the two control litters that had missing temperature logs for the cool phase. This model produced similar results, indicating that T_a did not significantly contribute to the between condition difference in pup activity levels on P8 (see Table S13).

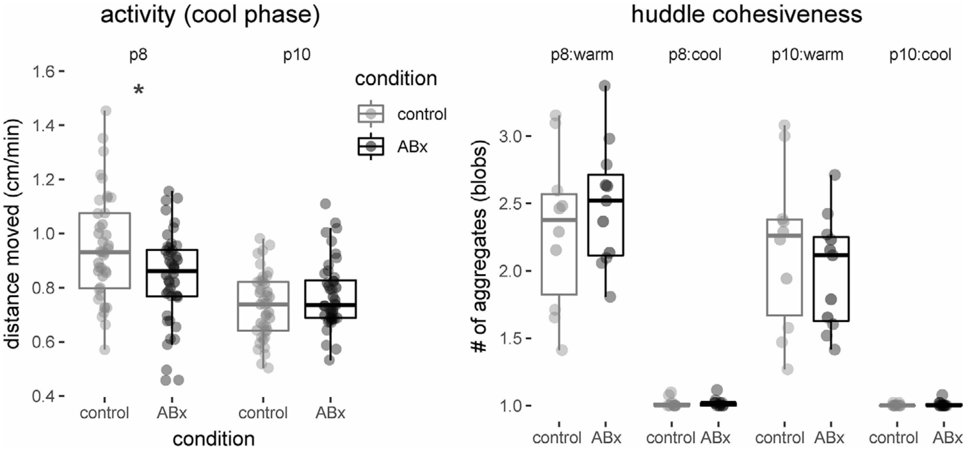

FIGURE 5 Activity level and cohesiveness while huddling as a function of age and dam treatment condition (i.e., control vs. ABx-treated). The left panel depicts activity level during the cool phase of the huddling test, quantified as the average distance moved (cm/min) by pups in the huddle. The right panel depicts huddle "cohesiveness" during the warm versus cool phases of the test, quantified as the average number of aggregates or "blobs" present. A score of 1 on the latter scale would indicate that all pups are in contact, that is, forming a single aggregate. A score of 4, in contrast, would indicate that all pups were isolated, not making any contact with each other. An asterisk (*) denotes significance at $p < .05$

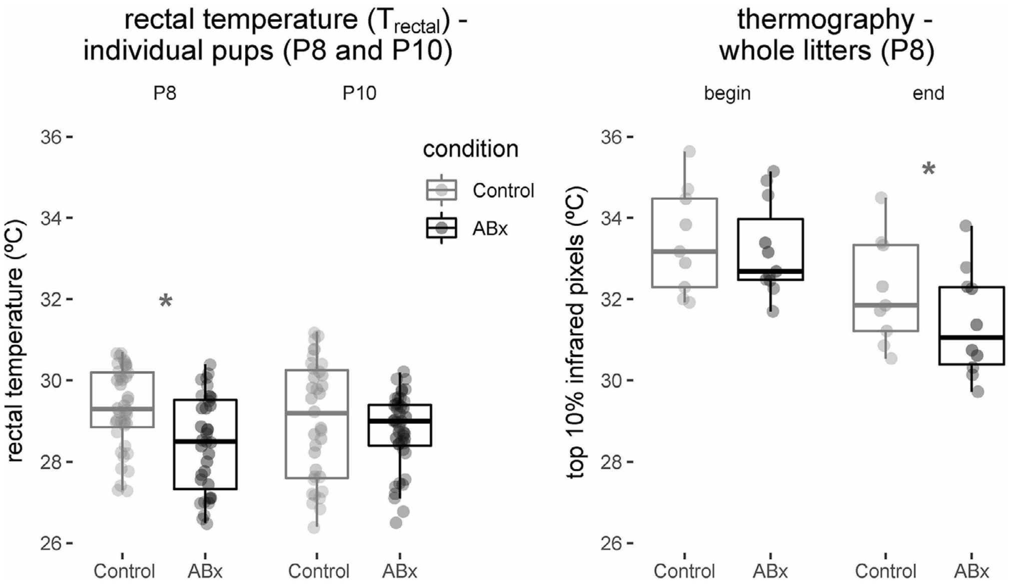

FIGURE 6 Measures of thermal homeostasis obtained during the huddling test on P8 and P10 as a function of dam treatment condition (i.e., control vs. ABx-treated). The left panel depicts rectal temperature (T_{rectal}) taken immediately after the huddling test on P8 and P10. The right panel shows a whole-litter measure of temperature regulation, obtained using the average temperature value for the top 10% of pixels in thermographs captured at the beginning versus end of testing on P8. The measure was not employed on P10 due to the large increase in fur development between P8 and P10. A double asterisk (**) denotes significance in post hoc testing at $p < .05$, whereas a triple asterisk (***) denote significance at $p < .001$

4 | DISCUSSION

Gut microbiome–brain interactions, both as a construct (Boem et al., 2021; Martin et al., 2018; Sarkar et al., 2018) and as a constellation of phenomena (Cusick et al., 2021; Cussotto et al., 2018; Nagpal & Cryan, 2021), have stimulated vigorous activity in basic, translational, and clinical research. Among the many remarkable findings are data suggesting links between early life ABx exposure, GI issues—including dysbiosis—and ASDs (e.g., Adams et al., 2007; Atladóttir et al., 2012; Axelsson et al., 2019b; Bittker & Bell, 2018; Guisso et al., 2018; Hisle-Gorman et al., 2018; Vargason et al., 2019). Microbiome–brain–ASD linkages are nevertheless multiply confounded given that individuals with ASD often experience a higher burden of sickness and thus ABx exposure early in life (e.g., Brown, 2012; Niehus &

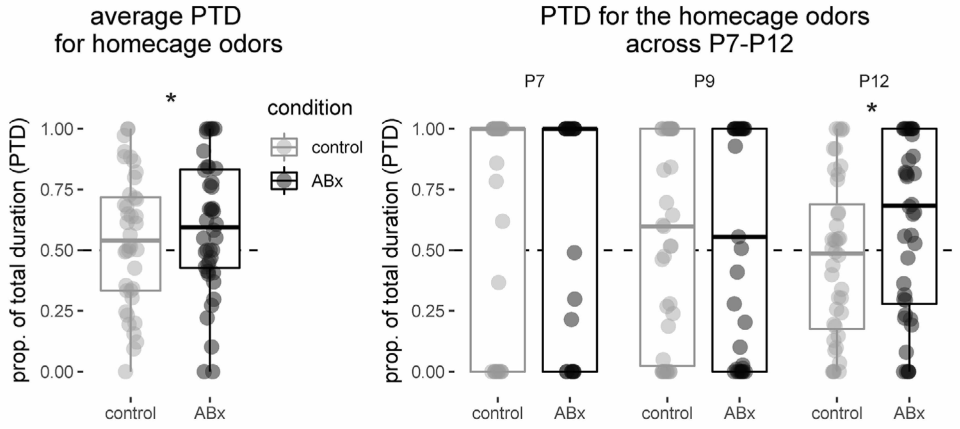

FIGURE 7 Proportion of total duration (PTD) for the homecage odor side during homecage odor preference test. Scores above .5 indicate a preference for the homecage side, whereas scores below .5 indicate more time spent on the unfamiliar nest side of the apparatus. The left panel illustrates the significant main effect of condition (i.e., pups from control vs. ABx-treated dams) on PTD scores for the homecage odor side, with scores averaged across ages. The right panel shows scores over time, with substantial variability at P7 and P9 and a significant difference between pups of ABx-treated and control dams only at P12. A single asterisk (*) denotes significance in post-hoc testing ($p < .05$)

Lord, 2006; Rosen et al., 2007). A recent study also found that an abnormally restrictive dietary selection substantially explains microbiome abnormalities in ASDs (Yap et al., 2021). Animal models are thus needed to disentangle these many confounding variables and alternate mechanisms and pathways. Here, we examined how exposure of pregnant and nursing C57BL/6 mice to nonabsorbable ABx in drinking water from G12 to P14 affected the behavior of offspring early in life, using a modified version of the Tochitani et al.'s (2016) ABx regime. We then examined a range of normative social and "communicative" behaviors expressed by infant mice while concurrently measuring and controlling for thermoregulatory differences in these animals.

We found that exposing dams to ABx during the perinatal period significantly reduced microbial alpha diversity in fecal samples both in treated dams and their untreated offspring (Figure 2), with the latter effect being more robust in male offspring (lower panel of Figure 2). ABx treatment nevertheless did not alter overt markers of maternal health, including weight and fluid consumption (Figures S1 and S2, respectively). ABx treatment also had no impact on the probability that pregnant dams gave birth to live litters or on the makeup or survival of those litters. Moreover, markers of offspring physical growth, including weight (Figure S3) and tail length, did not differ significantly between offspring of control dams. Nevertheless, we found a number of significant behavioral and physiological differences relative to offspring of control dams, summarized in Table 1.

First, in brief (5 min) tests of maternal separation on P7 and P9 we found that offspring of ABx-treated dams tended to produce more 50–60 kHz USVs than offspring of control dams (Figure 3). This effect, though short of statistical significance, appeared to be the result of thermoregulatory differences in offspring of ABx-treated versus offspring of control dams. In particular, P7 pups from ABx-treated dams were significantly cooler than offspring of control dams, an effect that was observed across both rectal temperature (T_{rectal}) and interscapular temperature (T_{IS}) at the end of the 5 min test ($T_{\text{IS}}^{\text{end}}$), the latter quantified via thermography (see Figure 4). When T_{rectal} was added to the LME model for USVs significant condition × T_{rectal} interaction was found, involving significant negative correlation between T_{rectal} and USVs for pups of control dams that was absent in pups of ABx dams (see Figure S6). As illustrated in the lower panel of Figure 3, the effect of dam ABx treatment on offspring USVs was also highly sensitive to pup weight. In particular, there tended to be a slight positive relationship between weight and USV production for pups from control litters (i.e., heavier pups vocalizing more/lighter pups vocalizing less) that was significantly reversed in pups from ABx-treated dams.

Huddling is a critical behavior in rodents that serves both social and thermoregulatory functions during early development (Alberts, 1978; Harshaw & Alberts, 2012; Harshaw et al., 2018). In the present study, offspring of ABx-treated dams were significantly less active in response to cold than offspring of control dams on P8, though they did not differ in the overall cohesiveness of their huddles (see Figure 5). In addition, pups from ABx litters were, again, significantly cooler than pups from control litters, this time after huddling in response to cold—as shown by significantly lower T_{rectal} values on P8 (see Figure 6). As with the P7 thermal difference, this effect was confirmed by infrared thermography—in this case, an analysis of whole group temperature regulation extracted from thermographs (see Figure 6). As there were no baseline differences in body temperature and no differences in huddle cohesiveness, it appears that pups from ABx litters *cooled more rapidly* during the huddling test than pups from control litters. Ostensibly small differences in individual activity and body temperature can, over time, lead to large thermal and energetic differences in the context of a huddle (Alberts, 1978; Gilbert et al., 2012) in addition to behavioral changes such as assortment by sex (Harshaw et al., 2014; Harshaw

TABLE 1 Summary of linear mixed effects (LME) model results for physiological and behavioral outcomes

Assay			Sig. effect		
Outcome	**Weight**†	**Sex**ψ	**Age**@	**ABx**	**ABx × T_{rectal}**
Maternal sep.					
USVs		**	**	††, ‡	
T_{rectal}	**, @		***	@	
T_{IS}			***	**, ψ, @, ☆	
USVs ← T_{rectal}		**	**	††	*
USVs ← T_{IS}		**	**	†	
Huddling					
Activity			***	@@	
Activity ← T_a			***	@@	
Cohesiveness			*		
T_{rectal}^{end}	*			@@@	
T_{huddle}	**(weight × phase)			*, †, *(weight × phase × ABx)	
Home odor pref.					
Activity			***		
Latency home			***		
Duration home				*	

*Indicate a significant main effect of a given predictor.
†Indicate a significant two-way interaction with pup weight (i.e., Z score, normalized within sex).
@Indicate a significant two-way interaction with pup age.
ψIndicate a significant two-way interaction with pup sex.
‡Indicate a significant three-way interaction with pup weight and age.
☆Indicates a significant three-way interaction with pup sex and age.
*Indicates an effect significant at $p < .05$.
**Indicates an effect significant at $p < .01$.
***Indicates significance at $p < .001$.

& Alberts, 2012) and differences in contact interactions among pups (Bautista et al., 2010; Harshaw et al., 2018; Sokoloff & Blumberg, 2001).

Finally, we assessed pups' olfactory-guided home orientation, that is, their tendency to preferentially approach and remain in proximity to homecage odors, over the course of the early pre-weaning period. This test has been used with numerous lab species, including rats (Gregory & Pfaff, 1971), hamsters (Devor & Schneider, 1974), and mice (Meyer & Alberts, 2016). This olfactory preference is often interpreted as functioning as a "tether" to keep young pups in or near the nest. Typically, a *developmental dissolution* of this olfactory tether occurs prior to weaning that facilitates older pups' egressions from the nest and affords opportunities to explore new areas of the environment and be exposed to new experiences. In a previous study in mice, the olfactory tether to home cage odor was strongly present in pups at P6 but waned by P12 (Meyer & Alberts, 2016). In the present study, pups from both ABx and control litters showed inconsistent preferences at P7 and P9. At P12, however, pups from ABx-treated dams showed a clear bias toward homecage odors that was significantly greater than that shown by the offspring of control dams (see Figure 7). This pattern can be interpreted as a delay in social development, in which dissolution of a behavioral attraction to a social cue (home-associated odors) is abnormally maintained and exposures to new social experiences are delayed or diminished.

Overall, the present research benefited from systematic attention to thermal dimensions of testing and of pups' thermoregulatory capabilities (cf. Blumberg, 2001). We controlled ambient temperature, monitored rectal and body surface temperatures, and included temperature-related variables such as sex and body mass. As in our prior studies (e.g., Harshaw et al., 2014; 2018) we detected relationships among sex, body mass, behavior, between brown adipose tissue thermogenesis and more general measures of body temperature, particularly during cold challenge.

Here, we found several differences in offspring behavior following maternal ABx treatment and microbiome disruption. This included differences in activity level in response to cold, modulation of USV production during maternal separation, and attraction to homecage odors in homecage odor preference tests. We also found significant differences in temperature regulation in the offspring of ABx-treated dams at younger (i.e., P7 and P8) relative to older (i.e., P9 and P10) ages. More specifically, multiple measures (i.e., rectal and thermographic) indicated impaired thermoregulatory ability or *more rapid cooling* in offspring of ABx-treated dams. Given that there are a number of potential reasons why this thermal difference may have normalized in only slightly older pups—e.g., pups undergo significant fur development over this period—the question deserves further attention, as does the more general question of metabolic effects of maternal ABx treatment on offspring (e.g., on oxygen consumption, lipid metabolism, accumulation of lipids in brown adipose tissue, etc.). Importantly, we confirmed that key, early-appearing social behaviors displayed by pups in both the maternal separation and home cage odor preference tests, were highly sensitive both to the thermoregulatory state of pups and between-condition differences in thermoregulation (cf. Blumberg, 2001). These findings add to a body of literature emphasizing the sensitivity of such measures to the thermal state of infant subjects (see Blumberg, 2001; Blumberg & Sokoloff, 1998; Harshaw & Alberts, 2012) and thus to the interactions that guide and modulate social development (e.g., de Medeiros et al., 2010; Faust et al., 2020; Hudson et al., 2011).

Commensal microbiota make a significant metabolic contribution to thermal homeostasis (Harrison & Hewitt, 1978; Kluger et al., 1990) and depletion of microbiota via ABx can thus reduce core body temperature (Conn et al., 1991; Fuller & Mitchell, 1999; Kluger et al., 1990). Recent studies have provided evidence of a significant role for gut microbiota in regulating metabolism (e.g., Krisko et al., 2020; Moriya et al., 2017; Suárez-Zamorano et al., 2015) and various components of cold adaptation, including brown adipose tissue thermogenesis (e.g., Chevalier et al., 2015; Nicholls et al., 2016; Zare et al., 2018). The results of the present study highlight that investigations of the effects of early life microbiome perturbation, including perturbation via ABx, must consider the thermal and metabolic effects of these manipulations when interpreting behavioral outcomes, even when those outcomes appear unrelated to temperature homeostasis. It must be emphasized that this issue is not restricted to work on infants, as a growing number of studies have highlighted that the temperatures at which adult mice are reared and tested place these animals in a state of near-constant cold stress (see Fischer et al., 2018; Hankenson et al., 2018; Hylander & Repasky, 2016).

There are limitations to the current study which deserve to be noted and addressed in future experiments. In the present study, the ABx exposure period (i.e., G12–P14) was lengthy and did not separate pre- from post-natal effects of maternal ABx treatment. Our goal was to disrupt both the maternal microbiome and transfer of microbiota from mother to offspring using a nonabsorbable ABx regimen, rather than closely mimic ABx treatment as might occur in a typical human pregnancy. The experiments described here thus represent a first step—a proof of concept—that can be used to develop a model with more direct translational potential. Another limitation of the present study is a lack of long-term outcomes in offspring. Our focus here was on early-appearing social behaviors, given that ASDs are developmental disorders that can be detected fairly early in postnatal development.

This study adds to a rapidly growing literature demonstrating that ABx treatment can lead to significant shifts in behavior in rodent models, whether exposure occurs in early life (e.g., Leclercq et al., 2017; Perna et al., 2021; Tochitani et al., 2016) or adulthood (e.g., Ceylani et al., 2018; Desbonnet et al., 2015; Gacias et al., 2016; Sylvia et al., 2017). We also demonstrated, for the first time, that maternal ABx treatment can significantly impact thermoregulation in unexposed offspring. We also demonstrated that such thermoregulatory impairments can significantly affect early-appearing social behaviors in infancy (cf. Blumberg, 2001), including tests of maternal separation and homecage odor preference. When combined with findings showing that ABx can cause significant reductions in body temperature in adult rodents, our results make clear that future studies of ABx-induced behavioral change must incorporate both thermal/metabolic measures and careful control of ambient conditions to ensure that behavioral differences observed are not simply a byproduct of metabolic changes in these models. Such control will permit more precise studies of underlying neural, immunological, and endocrine mechanisms while undoubtedly leading to greater replicability of findings in this burgeoning area of research.

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CONFLICT OF INTEREST

The authors have no conflicts to report.

DATA AVAILABILITY STATEMENT

All data reported here will be made available upon request.

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