Got Milk? Maternal immune activation during the mid-lactational period affects nutritional milk quality and adolescent offspring sensory processing in male and female rats

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Abstract

The neonatal environment requires a high level of maternal demand in terms of both breastfeeding and other forms of maternal care. Previous studies have underscored the importance of these maternal factors on offspring development and behavior. However, their contribution as dynamic variables in animal models of early life stress are often overlooked. In the present study, we show that lipopolysaccharide (LPS)-induced maternal immune activation (MIA) on postnatal day (P)10 immediately elevated milk corticosterone concentrations, which recovered by P11. In contrast, both milk triglyceride and percent creamatocrit values demonstrated a prolonged decrease following inflammatory challenge. Sustained inflammatory-induced changes to the nutritional quality of milk were also evidenced by its composition of microbial communities associated with inefficient energy and lipid metabolism. Nutritional deficits in early development have been associated with metabolic dysfunction later in life. Indeed, MIA-associated changes in the nutritional profile of milk were reflected by increased adolescent offspring bodyweights. While MIA did not decrease maternal care quality, there was a significant compensatory increase in maternal licking and grooming the day that followed the inflammatory challenge. However, this did not protect against disrupted neonatal huddling or later-life alterations in sensorimotor gating and mechanical allodynia in MIA offspring. Animal models of early life stress can impact both parents and their offspring. One mechanism that can mediate the effects of such stressors is changes to maternal lactation quality which our data show can confer multifaceted and compounding effects on neonatal physiology and behavior.

1. Introduction

Epidemiological studies have shown that prenatal exposure to maternal immune activation (MIA) is a leading risk factor for developing psychiatric disorders such as autism and schizophrenia (Mahic et al., 2017; Meyer, 2019). Animal models of MIA often employ the bacterial endotoxin lipopolysaccharide (LPS) or the toll-like 3 receptor agonist polyinosinic:polycytidylic acid (Poly I:C) to elicit a maternal immune response (Mueller et al., 2019). Administration of either of these immunogens promotes a cascade of inflammatory cytokines in the host, such as tumor necrosis factor (TNF)-α, interleukins, and T cells, in addition to activation of the hypothalamic-pituitary-adrenal (HPA) axis (Kentner et al., 2018; Brown et al., 2009; Arsenault et al., 2014; Estes & McAllister, 2016). In concert, these physiological responses contribute to a variety of sickness behaviors, including ptosis (eye drooping), piloerection (raised and ruffled fur), lethargy, and reduced food intake (Connors et al., 2014; Arsenault et al., 2014). In addition to eliciting these acute sickness behaviors, LPS exposure can lead to other changes in both pregnant and non-pregnant animals such as reduced locomotion (Millett et al., 2019), anhedonia, and improved performance in the Barnes Maze and novel object recognition task (Dockman et al., 2021). While the sickness-inducing effects of immunogens are also recognized to affect the long-term development of offspring when exposed prenatally (Kentner et al., 2018; Estes & McAllister, 2016), the impact of maternal infection during lactation on mothers and their offspring are relatively unexplored. This is surprising because infections during the postnatal period are common, particularly in low resource settings, and can result in severe morbidity and mortality (Karolinski et al., 2010; Kutlesic et al., 2017; John et al., 2017; Hussein et al., 2011).

When administered to rodent dams during the lactational period, LPS can contribute to deficits in maternal care. This has been evidenced by reductions in the efficient arched back nursing posture and the number of rat pups retrieved and returned back to the nest (Vilela et

al., 2013). In line with the behavioral alterations, MIA in lactating dams may influence maternal milk quality. For example, maternal LPS treatment on postnatal days 4, 11, and 18 reduced the mRNA expression of milk nutrient precursors such as glucose and fatty acid transporters in the rat mammary gland (Ling & Alcorn, 2010). Exposure to MIA during the lactational period can also impact offspring behavior. For example, Nascimento and colleagues (2015) observed a reduction in ultrasonic vocalizations in male neonates shortly after their dams were treated with LPS. Although this presumably involved changes in various forms of maternal input signals (e.g., parental care and/or milk quality), the mechanisms of this MIA-derived effect on offspring behavior remain unclear,

Other exogenous factors have been shown to alter contents in breastmilk and subsequent offspring development (Lesorogol et al., 2018; Mäkelä et al., 2013; Chen et al., 2017; Grazia Di Benedetto et al., 2020; Edwards et al., 2021). For example, maternal high fat diet has been associated with offspring obesity alongside greater triglyceride and protein levels in milk (Franco et al., 2012). Psychological stress in humans reduces milk microbial diversity (Browne et al., 2019), which may have clinical implications for infant development. Indeed, maternal milk quality and the microbiota are critical to programming infant metabolism, gut microbiome, and the gut-brain axis (Badillo-Suárez et al., 2017; Sheard & Walker, 1988; Pannaraj et al., 2017; Anderson et al., 2014). Further, cortisol in maternal milk was found to predict temperament and promote greater body weights in infant primates, although milk energy content was also associated with body weight (Hinde et al., 2014). Together, this evidence suggests that 1) disturbances to the neonatal environment, such as MIA, alters the developmental trajectory of offspring, and 2) these alterations are mediated by changes in maternal care and/or nutritional milk quality. To explore these ideas, we assessed the effects of MIA challenge during the mid-lactational period on maternal care, maternal milk composition, and offspring behavior in Sprague-Dawley rats.

2. Materials and Methods

2.1. Animals and Housing

Male and female Sprague-Dawley rats (Charles River, Wilmington, MA) were housed in same-sex pairs and maintained at 20°C on a 12 h light/dark cycle (0700-1900 light) in standard sized laboratory cages (27×48×22 cm). Food and water were available *ad libitum* throughout the study. Animal procedures were conducted in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care with protocols approved by the Massachusetts College of Pharmacy and Health Sciences (MCPHS) Institutional Animal Care and Use Committee. **Figure 1A** outlines the experimental procedures and groups evaluated in this study. During breeding, animals were placed into larger cages (51 x 41 x 22 cm) and bred using a 1 male:2 female design. Pregnancy was confirmed by continued weight gain and visible teats during the later phase of gestation. Approximately two days prior to parturition, females were individually housed in fresh clean standard sized cages. Day of birth was designated as postnatal day (P)0 and litters were standardized to 10 pups per litter on P1. Wherever possible, litters were balanced with equal numbers of male and female pups. Animals stayed in this condition until they were weaned into same-sex pairs on P22, again using standard sized laboratory cages. All cages maintained a tube, a Nylabone chew toy®, and Nestlets® throughout the study, except during the two-day period prior to parturition through until P14 when toys were removed to protect pups from injury. Additional Nestlets® were available to litters during this time. One male and one female offspring from each litter were evaluated on behavioral tasks beginning on P43. The behavioral monitoring equipment

was cleaned thoroughly with the disinfectant cleaner Quatriside TB (Pharmacal Research Laboratories, Inc) between each animal and test. The inter-rater reliability between blinded scorers was found to be greater than r=0.80 for each observational measure reported in this study.

2.2. Maternal Immune Activation (MIA)

On the morning of P10, dams were removed from their litter and placed into a fresh clean cage located in a separate procedure room. To model MIA during the lactational period, dams were weighed and intraperitoneally (i.p.) administered either 100 μg/kg of the inflammatory endotoxin, lipopolysaccharide (LPS; Escherichia coli, serotype 026:B6; L-3755, Sigma, St. Louis, MO, USA; n = 7) or pyrogen-free saline (saline; n = 8) at the start of the separation period. Animals remained in this cage for 2 hours until milking. The purpose of the separation period was to allow for maternal milk accumulation so samples could be collected at the height of the inflammatory response. In their regular holding room, pups were placed into a smaller clean cage positioned on top of a heating pad to maintain their body temperature. Pups were weighed immediately prior to being returned to their dams and again 2 and 24 hours later, alongside the inspection of milk bands, to monitor their health. Twenty-four hours following the P10 inflammatory challenge, milk collection procedures were repeated to evaluate sustained changes in milk quality. Additional details can be found in the methodological reporting table from Kentner et al. (2019), provided as **Supplementary Table S1**.

2.3. Milk Sample Collection and Composition

Milk was collected from each dam on P10 and P11 following a modified procedure from Paul et al. (2015). Immediately following each 2-hour separation period, dams were lightly anesthetized with isoflurane in O2, followed by the administration of 0.2 mL of oxytocin (20 USP/mL i.p.). The pharmacokinetic profile of isoflurane suggests that the drug is not absorbed by offspring and that breastfeeding can resume immediately after anesthesia (Drugs and Lactation Database, 2020; Lee & Rubin, 1993). Similarly, oxytocin is not expected to affect offspring given its short plasma half-life of 1-6 minutes that is reduced even further during lactation (Par Pharmaceutical Inc, 2020). Teats were prepared by moistening the collection areas with distilled water. Milk was obtained from each dam by gently squeezing the base of the teat and manually expelling the milk for collection. At the time of milk collection, ~20 ul of sample was collected into a microhematocrit tube, which was then sealed and placed into a hematocrit spinner (2 minutes at 13, 700 g; StatSpin CritSpin Microhematocrit Centrifuge, Beckman Coulter, Inc). Measurements were taken to calculate percent (%) creamatocrit by evaluating the sample separation into each of the cream and clear layers by following the procedures outlined in Paul et al. (2015). The remaining milk that was collected (~500 ul per animal) was aliquoted across microcentrifuge tubes and stored at -80°C until further processing. Collection time took about 10-25 minutes per rat, and dams were reunited with their litters in their home cage as soon as they fully awoke from anesthesia.

Milk samples were homogenized by overnight rotation on a Mini Tube Rotator (Fisher Scientific Cat. #88861051) at 4°C before analysis. A lactose assay kit (Sigma-Aldrich Cat. #MAK017) was used to measure lactose content in milk diluted 1:500 with lactose assay buffer (as outlined by Chen et al., 2017). Triglycerides were evaluated using a colorimetric assay kit (Abcam, Cat. #ab65336) at 1:1000 dilution. The Pierce™ BCA Protein Assay Kit

(Cat. #23227) was run at a 1:50 dilution to measure protein levels in milk. The small sample assay protocol of the corticosterone ELISA kit (#ADI-900–097, Enzo Life Sciences, Farmingdale, NY) was followed, as recommended by the manufacturer, using a 1:40 dilution. Milk levels of immunoglobulin (Ig) A were assessed with an assay kit (Bethyl Laboratories, Cat. # E111-102) using samples diluted to 1:1000 while interleukin (IL)-6 (Thermo Fisher Scientific, Cat. #BMS625) was evaluated using samples diluted 1:2. Manufacturer's instructions were followed for all ELISA kits (n = 7).

P10 (n = 6) and P11 (n = 6-7) milk samples underwent microbiome sequencing. DNA extraction was performed using the ZymoBIOMICS ®-96 MagBead DNA Kit (Zymo Research, Irvine, CA) and 16S targeted sequencing was completed using the *Quick*-16S™ NGS Library Prep Kit (Zymo Research, Irvine, CA) and V3-V4 16S primers (Zymo Research, Irvine, CA). The sequencing library was prepared using real-time PCR. Final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned with the Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA) which was then quantified with TapeStation® (Agilent Technologies, Santa Clara, CA) and Qubit® (Thermo Fisher Scientific, Waltham, WA). The final library was sequenced on Illumina® MiSeq™ with a v3 reagent kit (600 cycles) and sequencing was performed with 10% PhiX spike-in. Unique amplicon sequence variants were inferred from raw reads using the DADA2 pipeline (Callahan et al., 2016). Potential sequencing errors and chimeric sequences were also removed with the DADA2 pipeline. Taxonomy assignment was performed using Uclust from Qiime (v.1.9.1) and referenced with the Zymo Research Database (Zymo Research, Irvine, CA).

2.4. Neonatal Huddling Behavior

Two-hours after being reunited with their dams on P10 and P11, male and female offspring were quickly weighed and evaluated on their huddling behavior (n = 7-8 full litters). This paradigm was adapted from Naskar and colleagues (2019). Briefly, litters were uniformly placed along the perimeter of a 40 cm x 40 cm area and video recorded for 10 minutes. The average number of pup clusters (2 or more pups in physical contact) was determined by extracting one video frame every 30 seconds and averaging the number of clusters observed across 20 frames. The average time spent together was determined by calculating the average time (in seconds) each pup spent in a clump for the entire 10-minute video.

2.5. Maternal Behavior

To evaluate whether lactational MIA exposure affected maternal behavior, we monitored passive maternal care between P10 and P11 (n = 7-8). The first observation took place on the afternoon of P10 (15:00hrs), following the inflammatory challenge. Maternal care observations took place again at 20:00hrs, and then at 07:30hrs on P11. Following the procedures of Strzelewicz et al (2021; 2019), each session consisted of six observations and a total composite score was calculated for each of the morning, afternoon, and evening time points. Dams were evaluated for 1-minute intervals per observation, with at least 5 minutes of no observations occurring between each of the 1-minute bins. Maternal care observations recorded included the frequency of pup-directed behaviors (i.e., dam licking/grooming pup, active/high crouch nursing, passive/low crouch nursing, pup retrieval), self-directed behaviors (i.e., dam eating/drinking, dam self-grooming), and nest building/digging behavior. Total time the dam spent on nest (seconds) was also recorded.

2.6. Adolescent Open Field and Social Behavior

On P43, male and female offspring were habituated to an open field arena for five-minutes (40 cm × 40 cm × 28 cm; Duque-Wilckens et al., 2020; Williams et al., 2020; n = 7-8). An automated behavioral monitoring software program (Cleversys TopScan, Reston, VA) was used to evaluate animals on their duration spent (seconds) and frequency of crosses made in each of the center and perimeter of the arena. Percent time spent in the center of the arena and total distance traveled (cm) were also evaluated.

Immediately following the open field habituation period, two clean wire cups were placed on opposite ends of the arena to evaluate social preference (adapted from Crawley, 2007). One cup contained a novel object, and the other cup contained a novel rat of the same sex, age, and strain. Placement of the cups were counterbalanced between trials. Using a manual behavioral monitoring program (ODLog™ 2.0, http://www.macropodsoftware. com/), active investigation was recorded when an experimental rat directed its nose within 2 cm of a wire cup, or it was touching the cup. A social preference index was calculated by the formula ([time spent with the rat] / [time spent with the inanimate object + time spent with the rat]) - 0.5 (Scarborough et al., 2020).

2.7. Mechanical Allodynia

The next day, each animal was acclimatized for 30 minutes to an acrylic cage, with a wire grid floor. Mechanical allodynia was assessed using a pressure-meter which consisted of a hand-held force transducer fitted with a polypropylene rigid tip (Electronic von Frey Aesthesiometer, IITC, Inc, Life Science Instruments, Woodland Hills, CA, USA). The polypropylene tip was applied vertically to the central area of the animal's left hind paw with increasing force. The trial ended when the rat withdrew their paw from the tip, at which point the intensity of the stimulus was automatically recorded by the electronic pressure-meter. The average of four test trials was calculated as the mechanical withdrawal threshold (grams; Yan & Kentner, 2017).

2.8. Prepulse Inhibition of the Acoustic Startle Reflex

Three hours following the evaluation of mechanical allodynia, animals were placed into startle chambers and evaluated on prepulse inhibition (PPI) of the acoustic startle reflex (San Diego Instruments, San Diego, CA, USA). Sessions each consisted of pulse-alone, prepulse-plus-pulse and prepulse-alone trials, as well as no-stimulus trials in which only a background noise of 65 dB was presented Giovanoli et al., (2013). The acoustic startle software administered one 40-ms pulse of white noise (120 dB) in combination with one of five different prepulses. The prepulses were made up of a 20-ms burst of white noise at five different intensities (69, 73, 77, 81, 85 dB), corresponding to 4, 8, 12, 16, and 20 dB above the background noise). After a startle habituation phase, each trial stimulus was pseudorandomly presented 12 times. The average interval between successive trials (ITI) was 15 ± 5 sec. Sessions terminated with 6 consecutive pulse-alone trials. PPI was calculated as percent inhibition of the startle response obtained in the prepulse-plus-trials compared to pulse-alone trials: [1-(mean reactivity on prepulse-plus-pulse trials / mean reactivity on pulse-alone trials) × 1/100] and expressed as % PPI for each animal at each of the five possible prepulse intensities.

2.9. Conditioned Fear

Fear conditioning has been shown to modulate PPI of the acoustic startle reflex (Ishii et al., 2010; Balogh et al., 2002). At the conclusion of the PPI session, animals were allowed to rest for 2 hours before commencing Day 1 of fear conditioning trials. On Day 1, animals received 10 trials of a light stimulus (CS) paired with a 0.6 mA foot shock (US). The next day (Day 2), animals completed the PPI task for a second time, with half of the pseudorandom trials beginning with the presentation of the CS in order to assess how the element of fear influences PPI of the acoustic startle reflex. Percent PPI was calculated (described above) for trials with and without the CS.

2.10. Tissue Collection

On P46, a mixture of ketamine/xylazine (150 mg/kg, i.p./50 mg/kg, i.p.) was used to anesthetize animals. Tissues were dissected, frozen on dry ice, and stored at −80°C for future processing.

2.11. Statistical analyses

Statistics were performed using the software package Statistical Software for the Social Sciences (SPSS) version 26.0 (IBM, Armonk, NY) or GraphPad Prism (version 9.0). Because the dataset was not powered to evaluate sex-differences directly, male and female animals were evaluated separately (Ordoñes Sanchez et al., 2021). Data are depicted as each sex separately and collapsed across sex for display purposes. Two-way repeated measure ANOVAs (MIA x Time) were used to evaluate milk and behavioral measures across P10 and P11. Violations to the assumption of sphericity were addressed using the Huyndt Feldt correction.

One-way ANOVAs were used as appropriate for all other measures unless there were violations to the assumption of normality (Shapiro-Wilk test) in which case Kruskal-Wallis tests were employed (expressed as X^2). The von Frey threshold data was assessed using body weight as a covariate. Data are graphically expressed as mean ± SEM. The partial eta-squared (n_p^2) is also reported as an index of effect size for the ANOVAs (the range of values being 0.02 = small effect, 0.13 = moderate effect, 0.26 = large effect; Miles and Shevlin, 2001).

For microbiome sequencing, samples underwent composition visualization, in addition to alpha-diversity and beta-diversity analyses using Qiime (v.1.9.1) and statistical comparisons were performed using Kruskal-Wallis (Caporaso et al., 2010). Linear discriminant analysis effect size (LEfSe; http://huttenhower.sph.harvard.edu/lefse/) was utilized to determine significant differences in taxonomy abundance between each group as previously described (Segata et al., 2011; Schellekens et al., 2021). Briefly, LEfSe uses a series of nonparametric tests to create a model that identifies taxa that are most likely to explain differences between experimental groups (Segata et a., 2011).

3. Results

3.1. MIA challenge affected maternal care and offspring huddling behavior

Immune challenge during lactation significantly impacted the number of maternal care bouts directed towards pups. For example, a MIA by time interaction was identified for the number of licks pups received following maternal LPS exposure ($F(2, 26) = 31.435$, $p =$

0.001, n_p^2 = 0.701; **Figure 1B**). While the number of licks received was not significantly affected on P10 (p >0.05), there was a general pattern of reduced care provided by MIA treated dams on the afternoon of P10 (p = 0.063). This was followed by a rebound of maternal pup licking by MIA dams on the morning of P11, possibly to compensate for overall reduced maternal care due to illness (p = 0.001). The display of both active and low crouch nursing behaviors was affected by the circadian cycle (active: F(2, 26) = 7.752, p = 0.0022, n_p^2 = 0.374; low: F(2, 26) = 3.960, p = 0.032, n_p^2 = 0.233: **Figure 1C,D**), but not MIA experience (p>0.05). Neither time of day nor MIA affected the amount of time spent on the nest (p>0.05; **Figure 1E**). Maternal self-directed care was impacted as a function of sickness. There was a significant MIA by time interaction for total number of self-grooms (F (1.322, 26) = 12.33, p = 0.001, n_p^2 = 0.485; Huyndt Feldt correction; **Figure 1F**). MIA dams had fewer bouts of grooming on the evening of P10 (p = 0.025), which reversed substantially by the morning of P11 (p = 0.0001). The number of maternal eating bouts also seemed to rebound in a compensatory manner following MIA challenge, although there was no significant effect of time (p>0.05; Figure 1G). There was however, a main effect of MIA (F(1, 13) = 10.64, p = 0.006, n_p^2 = 0.450; **Figure 1G)**). Additional maternal care data are presented in **Supplementary Figure 1A-D**.

Huddling behavior was significantly reduced in the offspring of LPS treated dams compared to saline (main effect of MIA; time spent huddling: F(1, 12) = 30.079, p = 0.001, n_p^2 = 0.715; **Figure 1H**; average number of clusters: F(1, 12) = 8.33, p = 0.014, n_p^2 = 0.410; **Figure 1I)**. See **Figure 1J** for representative displays of huddling behavior in saline and LPS offspring.

3.2. MIA challenge impacted nutritional composition of milk and microbiome communities

For milk collection (see photographs in **Figure 2A** depicting the milk collection procedure), there was a significant MIA by time interaction for maternal milk levels of corticosterone (F(1, 13) = 8.496, p = 0.013, n_p^2 = 0.415; **Figure 2B**). This stress-associated hormone was elevated in milk samples 3-hours following MIA challenge ((F(1, 13) = 9.512, p = 0.009), and was recovered by P11 (p>0.05). A MIA by time interaction was also present for % creamatocrit (F(1, 13) = 5.86, p = 0.032, n_p^2 = 0.858; **Figure 2C**), which is linearly related to the fat concentration and energy content of milk (Lucas et al., 1978; Paul et al., 2015; see **Supplementary Figure 2A, B**). These measures were significantly lower in the milk of MIA exposed mothers compared to saline on P11 (p = 0.002). Triglycerides were also reduced in the milk of MIA treated dams on P11 (main effect of MIA: F(1, 13) = 6.496, p = 0.026, n_p^2 = 0.351; **Figure 2D**).

While inflammatory cytokine expression in milk does not reflect levels found in the blood, chronic in vitro stimulation of human breast milk cells with LPS can induce the expression of IL-6 (Hawkes et al., 2002). In order to examine the possibility that maternal LPS challenge increased expression of this cytokine that could be transferred to nursing offspring, we analyzed the concentration of milk IL-6. While there were no significant differences in IL-6 concentration between saline and MIA treated dams (p > 0.05), there was a main effect of time on this measure (F(1,13) = 33.01, p < 0.001, n_p^2 = 0.717; **Figure 2E).** Additional data on maternal milk composition (i.e., protein, lactose, and IgA concentrations) can be found in **Supplementary Figure 2C-E.**

Microbiome sequencing revealed no main effect of treatment group on alpha (**Figure 2F)** or beta diversity in milk samples (main effect of MIA: p > 0.05; **Figure 2G, H**). Milk samples collected on P11 demonstrated significantly greater alpha diversity along the Shannon index compared to samples collected on P10 (main effect of time: $X^2(1)$ = 5.33, p =

0.021; **Figure 2F**)). Between treatment groups, LEfSE analysis identified 9 differently abundant taxa on P10 and 6 significantly abundant taxa on P11 **(Figure 2I, J)**. These analyses revealed a greater abundance of *Pseudomonadaceae* (LDA score = 2.94, p=0.02) and *Christensenellaceae* (LDA score = 2.82, p = 0.02) on P10, and a greater expression of *Stenotrophomonas maltophilia* (LDA score= 3.45, p=0.025), *Ruminococcaceae* (LDA score = 3.35, p = 0.02) and *Lachnospiraceae* (LDA score= 3.85, p = 0.01) on P11 in the milk from MIA dams. For additional data on the composition of the milk microbiome, see **Supplementary Figures 3-6.**

3.4. The combination of MIA challenge and nutritional deficit affected adolescent offspring physiology and behavior.

Male, but not female, MIA offspring were significantly heavier than saline controls on P43 (One-way ANOVA main effect of MIA: males: $F(1,13) = 19.037$, $p = 0.001$, $n_p^2 = 0.594$; females: $p > 0.05$; **Figure 3A**). Using body weight as a covariate, female offspring from LPS treated dams were found to have higher thresholds in the von Frey test compared to their saline counterparts (females: $F(1, 12) = 8.925$, $p = 0.011$, $n_p^2 = 0.427$; males: $p > 0.05$; **Figure 3B**). This is suggestive of reduced sensitivity to tactile stimulation.

Although there were no differences in distance travelled (cm), percent time spent in the center, nor in the duration of time (seconds) spent in the center or perimeter of the open field arena ($p>0.05$; see **Supplementary Figure 7A-D**), male offspring of MIA exposed mothers displayed an increased number of crosses into both the center ($F(1, 13) = 5.744$, $p = 0.032$, $n_p^2 = 0.306$; **Figure 3C**) and perimeter ($F(1, 13) = 6.513$, $p = 0.024$, $n_p^2 = 0.334$; **Figure 3D**) of the arena (females: $p > 0.05$). Social preference was not affected by the early life experience for either sex ($p > 0.05$; **Figure 3E**).

Sensorimotor gating was evaluated using PPI of the acoustic startle reflex. A significant MIA by time interaction (males: $F(4, 52) = 4.578$, $p = 0.003$, $n_p^2 = 0.260$; **Figure 3F**) and a significant main effect of time (females: $F(4, 52) = 80.357$, $p = 0.001$, $n_p^2 = 0.861$; **Figure 3G, H** collapsed across sex for display purposes) confirmed that all groups demonstrated an increased % PPI as the intensity was raised from 69 to 85 dB. While females were unaffected ($p > 0.05$), male offspring originating from MIA exposed mothers had attenuated PPI values compared to saline controls at the 69dB ($F(1, 13) = 17.009$, $p = 0.001$, $n_p^2 = 0.743$), 73dB ($F(1, 13) = 5.927$, $p = 0.030$, $n_p^2 = 0.581$), and 81dB ($F(1, 13) = 8.315$, $p = 0.013$, $n_p^2 = 0.633$) intensities. The mean percent prepulse inhibition across all five prepulse intensities was not affected as a function of maternal MIA exposure ($p > 0.05$; **Figure 3I**).

With respect to conditioned fear, there was a main effect of drug for % PPI during the trials without fear for 69dB (females: $F(1, 13) = 12.969$, $p = 0.003$, $n_p^2 = 0.495$), 73dB (males: $F(1, 13) = 6.787$, $p = 0.022$, $n_p^2 = 0.343$), 81dB (males: $F(1, 13) = 6.787$, $p = 0.022$, $n_p^2 = 0.343$; females: $F(1, 13) = 25.117$, $p = 0.001$, $n_p^2 = 0.659$) and 85 dB (males: $F(1, 13) = 13.124$, $p = 0.003$, $n_p^2 = 0.502$; **Figure 3J, K, L** collapsed across sex for display purposes). % PPI for trials containing the CS was not significantly different between saline and MIA animals for either sex ($p>0.05$; **Figures 3M** collapsed across sex for display purposes). One-way ANOVA revealed a main effect of trial type ($F(4, 52) = 8.19$, $p=0.008$, $n_p^2 = .226$; **Figure 3N**), which was expected given that the added element of fear increases % PPI (Ishii et al., 2010; Balogh et al., 2002). Conditioned fear did not lead to significant changes in total mean % PPI when collapsed across all dB, regardless of drug or sex ($p > 0.05$; ; **Figure 3N** data collapsed across sex for display purposes), suggesting that fear modulated % PPI similarly regardless of sex or drug treatment.

4. Discussion

In the present study, we demonstrate that variations in milk quality may serve as part of a mechanism programming offspring physiology and behavior following stressors experienced during the mid-lactational period. For example, we show that MIA was associated with sustained changes in both the nutritional content and microbial profile of maternal milk. Moreover, the MIA-induced changes in milk fat and corticosterone levels were accompanied by physiological and behavioral alterations in offspring. For example, neonatal offspring from MIA-challenged dams spent less time huddling compared to controls. In adolescence, MIA offspring were heavier, demonstrated deficits in sensorimotor gating, and had increased mechanical allodynia thresholds. Importantly, these MIA-associated effects in offspring were not due to an overexposure of maternally derived IL-6, as milk concentrations of this cytokine were comparable between MIA and saline dams. Moreover, though a general pattern of reduced licking/grooming could be seen in MIA dams on P10, this effect was non-significant and care quality was overcompensated by P11. Rather, offspring behavior and bodyweight differences likely reflect the unique expression of the nutritional elements and metabolically relevant bacterial taxa found in P10 and P11 milk samples. While most of the MIA-related literature has been dedicated to uncovering the physiological and behavioral effects on offspring gestationally exposed to MIA, fewer studies have focused on the impact of MIA exposure after birth. The lactational period is an important time for neonates and here we underscore the value of maternal inputs (e.g., maternal milk quality) to offspring development.

MIA was associated with multiple changes in the composition of maternal milk content. We observed a significant increase in milk corticosterone 3-hours post LPS treatment that remitted by P11. Milk corticosterone is expressed linearly to plasma corticosterone (Patacchioli et al., 1992), validating our MIA model. Importantly, milk corticosterone can pass from a mother to her offspring where these glucocorticoids can remain active in the periphery and in the brain (Angelucci et al., 1985; Brummelte et al., 2010). Increased corticosterone exposure from breastmilk has previously contributed to HPA axis dysfunction as well as altered learning and memory abilities in rat pups of both sexes, independent of maternal care (Catalani et al., 1993; Catalani et al., 2002). In addition to its modulatory actions within the HPA axis, recent evidence has demonstrated the regulatory role of corticosterone in breastmilk, where the presence of this hormone (and the human equivalent, cortisol) can modulate nutritional elements including sodium, potassium, and fat (Zietek et al., 2021; Sullivan et al., 2011; Hinde et al., 2015). This evidence suggests that the temporary rise in corticosterone on P10 may have preceded downstream changes in milk nutritional quality. In line with this, MIA reduced % creamatocrit and milk triglycerides on P11. However, higher levels of glucocorticoids in milk alone are not a sufficient predictor of offspring bodyweight (Brummelte et al., 2010; Hinde et al., 2014), suggesting that the actions of corticosterone in neonates are likely interwoven with milk nutrition. Reductions in milk creamatocrit or triglycerides may instead have been due to direct actions of LPS. Indeed, previous studies have shown LPS to inhibit fat production in the mammary epithelial cells of cows (Liu et al., 2015; Chen et al., 2019) while LPS reduced fatty-acid transporter mRNA in the mammary gland of rats (Ling & Alcorn, 2010). It is unclear however, if inflammatory markers that follow LPS administration into the periphery are capable of crossing into maternal milk in vivo.

We measured levels of IL-6 in milk to investigate the possibility that maternally derived cytokines induced following MIA were being absorbed by offspring and affecting neurodevelopment. In prenatal models of MIA, IL-6 is a main proponent of changes in brain

development and behavior in offspring (Wu et al., 2017; Hsiao & Patterson, 2011). Moreover, in the presence of LPS, maternal milk cells can produce high amounts of IL-6 in vitro (Hawkes et al., 2002). The detection of IL-6 in our control samples was not surprising, given that IL-6 is involved in typical mammary gland functions (Basolo et al., 1996). However, we found no differences in milk IL-6 between the saline and MIA treatment groups, indicating that offspring behavioral changes were not programmed by greater exposure to maternal IL-6. Future studies must rule out the possibility that other inflammatory markers (e.g., TNF-α and other interleukins) may cross from maternal blood to milk, although it is unlikely (Hawkes et al., 2002). This evidence supports the idea that differences in nutritional quality, and not inflammatory profile, may be more influential in modulating offspring development and behavior in this postnatal MIA model.

Microbiome sequencing did not reveal significant differences in alpha or beta diversity between our saline and MIA treated dams. While general diversity was not affected, LEfSe analysis uncovered significant differences in the expression of certain taxa on P10 and P11. Specifically, MIA dams exhibited a higher abundance of bacterial families including *Pseudomonadaceae*, which are commonly over-expressed during infection (Hall-Stoodly, 2005). Surprisingly, MIA dams also exhibited higher levels of *Christensenellaceae*, which is associated with reduced inflammation and a lower expression of the proinflammatory lipopolysaccharide binding protein (LBP) in the gut (Citronberg et al., 2018; Citronberg et al., 2016). Significant increases in *Christensenellaceae* may have been compensatory to attenuate LBP-signaling and subsequent inflammatory responses. *Christensenellaceae* also negatively correlate with serum triglycerides (Waters & Ley, 2019), which may reflect the lower levels of triglycerides found the milk of our MIA dams. Further, milk from MIA dams also exhibited elevated levels of *Stenotrophomonas maltofilia*, a bacterium that thrives in the presence of LPS (Poroyko et al., 2015; Brooke, 2012). The increased expression of both *Lachnospiraceae* in the milk of LPS-treated MIA dams may in part explain the offspring bodyweight differences at P43 as this bacterium is associated with inefficient lipid and energy metabolism (Vacca et al., 2020; Palmas et al., 2021). Given that the microbes in breast milk directly colonize the infant gut (Sheard & Walker, 1988; Pannaraj et al., 2017), it is likely that these differently expressed taxa are being passed to the offspring to influence future metabolic processes. Additional studies are needed to explore potential therapeutic interventions, such as targeting the microbiome (Holingue et al., 2020) and the use of nutritional supplements, which may rescue changes in milk composition and subsequent offspring developmental outcomes.

We used several behavioral measures to explore the role of lactational MIA exposure on offspring development. Neonatal ‘huddling’ or ‘clumping’ is described as a form of social thermoregulation (Gilbert et al., 2009). One previous study found a reduction in nest seeking behavior in rat pups prenatally exposed to LPS (Baharnoori et al., 2012). Undernourishment has also been shown to reduce neonatal huddling in rat pups (Soriano et al., 2006), and huddling efficiency may be related serum triglycerides in newborn rabbits (García-Torres et al., 2015). This evidence suggests that MIA-modulated huddling may emerge due to differences in the fat content of maternal milk. Here, pups from MIA treated mothers displayed disorganized clumping behavior. Impairments in huddling have also been associated with delayed development of glutamatergic and GABAergic signaling in the prefrontal cortex and hippocampus (Naskar et al., 2019; Baharnoori et al., 2009), which may have long-term implications for offspring in adulthood. Indeed, deficits in neonatal huddling may be predictive of other behavioral alterations later in life (Schatz et al., 2018).

Prenatal MIA has been shown to modulate offspring performance on certain tasks, such as the open field, social preference, von Frey, PPI, and fear conditioning paradigms (Kentner et al., 2018; Fortier et al., 2007; Zhao et al., 2021; Inceoglu et al., 2006; Howland et al., 2012).

Here, we observed significant MIA induced reductions in % PPI that were exacerbated in male adult offspring. In addition, female MIA offspring demonstrated deficits in mechanical allodynia. These results, in addition to reduced neonatal huddling, point to generally impaired sensory processing in the offspring of dams exposed to MIA during lactation.

We found no differences between saline and MIA offspring performance in the fear conditioning or social preference paradigms, and MIA males exhibited less anxiolytic behavior as indicated by their increased number of crosses into the center of the open field task. While in sharp contrast to studies that have assessed gestational MIA, these results are not entirely surprising given the physiological differences between the pre and postnatal actions of MIA. For example, MIA in utero reorganizes the molecular construction of the placenta (Núñez Estevez et al., 2020; Hsaio & Patterson, 2011; Garay et al., 2013) and the results of the present study suggest lactational MIA targets milk quality and offspring nutrition. While complimentary, our results highlight the importance of the timing of MIA challenge to offspring developmental and behavioral programming.

Moreover, our MIA dams showed significant increases in pup-directed licking and grooming behavior the day following MIA challenge. The heightened maternal care demonstrated by MIA dams on P11 may have been a compensatory mechanism to buffer the effects of lactational MIA exposure. Tactile stimulation through the licking and grooming of pups is one form of maternal behavior that promotes brain development and modulates future offspring behavior (Champagne et al., 2003; Francis et al., 2002; Van Hasselt et al., 2012; Francis & Kuhar, 2008). Aside from a general pattern of decreased (but not significant) licking and grooming of pups on the afternoon of P10, we did not observe a drastic reduction in pup directed behaviors in MIA dams. The effects of LPS are highly dose dependent (Bison et al., 2009). While Vilela and colleagues (2013) observed more dramatic deficits in maternal care following MIA, they implemented a dose of LPS 5-times higher than the dose used in the present study. Moreover, lactational exposure to LPS using the same dose as that used in the current study also resulted in improved maternal care (Nascimento et al., 2015). Together, our results suggest that stressors experienced during the neonatal period may interact with maternal care and lactation quality, affecting offspring physiology and behavior. Indeed, early life stress models affect both the parent(s) and the offspring; the dynamic impacts on each need to be considered as part of the mechanistic programming of the developmental trajectory.

Conclusions

Maternal care and milk quality are important components to offspring development, brain health, and behavior. Here, we demonstrate that an acute maternal immune response during mid lactation is sufficient to trigger changes in breastmilk quality and its microbiome profile, in addition to the physiology and behavior of adolescent offspring. These results contribute to the broader literature by suggesting that the effects of MIA on offspring development are not restricted to the prenatal window. Moreover, this study ties in the characteristics of lactation and nutrition as part of a mechanism contributing to the trajectory of offspring development following early life stress. Given that animal models of early life stress can impact both parents and their offspring, the quality of maternal milk should be considered among the variables investigated in future studies. Finally, this work underscores the importance of research focused on potential therapeutic interventions (e.g., nutritional lactation supplements) and necessitates a better representation of pregnant and nursing people in both basic and clinical research.

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Author Contributions

H.D., H.T., A.C.K., ran the experiments; H.D., & A.C.K. analyzed and interpreted the data, and wrote the manuscript; A.C.K., designed and supervised the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figure Captions

Figure 1. Maternal care and offspring huddling behaviors following maternal immune activation (MIA) during the mid-lactational period. (A) Timeline of experimental procedures. Total number of maternal pup-directed behaviors (B) pup licks, (C) active nursing bouts, (D) low crouch nursing bouts, and (E) total time (seconds) that dam spent on nest. Total number of maternal self-directed behaviors (F) self-groom bouts and (G) eating bouts (n = 7-8). Maternal care was evaluated in the afternoon and night of postnatal day (P) 10 and the morning of P11. Pup huddling such as (H) total time spent huddling (seconds) and the (I) average number of clusters was evaluated across P10 and P11 following MIA (n = 7-8 full litters). (J) Shows representative photographs of the huddling behavior of saline and LPS litters. Data are expressed as mean ± SEM. *$p < 0.05$, ***$p < 0.001$, LPS versus Saline; $^{a}p < 0.05$, $^{aa}p < 0.01$, main effect of time; $^{b}p < 0.05$, $^{bb}p < 0.01$, $^{bbb}p < 0.001$, main effect of MIA.

Figure 2. Nutritional profile of milk and microbiome community distribution following maternal immune activation (MIA) during the mid-lactational period. (A) Photographs of maternal milk collection. Maternal milk levels of (B) corticosterone (ng/mL), (C) percent creamatocrit, (D) triglycerides (mg/dL), and (E) interleukin (IL)-6 (pg/mL) (n = 7-8). (F) Alpha diversity of P10 and P11 milk samples along the Shannon Index. (G) Beta diversity using principle coordinate analysis (PCoA) of P10 and (H) P11 milk samples were created using the matrix of pair-wise distance between samples determined by Bray-Curtis dissimilarity using unique amplicon variants. (I) Microbial composition of taxonomy at the family level for saline and LPS dams at P10 and P11 (n = 6-7). (J) LEfSe biomarkers plots. These figures display taxa that are significantly more abundant in the milk of saline-treated dams (blue bars) versus LPS treated dams (red bars) on P10 and P11. These taxa were identified based on their significant distributions ($p < 0.05$) and effect sizes (LDA score) larger than 2 (n = 6-7). Data are expressed as mean ± SEM. *$p < 0.05$, ***$p < 0.001$, LPS versus Saline; $^{a}p < 0.05$, $^{aa}p < 0.01$, main effect of time; $^{b}p < 0.05$, $^{bb}p < 0.01$, $^{bbb}p < 0.001$, main effect of MIA.

Figure 3. Adolescent offspring physiology and behavior following maternal immune activation (MIA) during the mid-lactational period. (A) P43 body weights (grams), (B) mechanical paw withdrawal thresholds (grams) on the von Frey test, number of crosses into the (C) center and (D) perimeter of an open field arena, and (E) social preference index.Line plots displaying percent prepulse inhibition (PPI) as a function of increasing prepulse intensities for (F) males, (G) females, and (H) males and females combined. (I) The bar plot shows the mean percent prepulse inhibition across all five prepulse intensities. Conditioned fear % PPI for (J) males, (K), females, and (L) males and females combined. (M) % PPI collapsed across both sex and drug for all five prepulse intensities. (N) Total % PPI collapsed across sex and all prepulse intensities to show trials without fear (black line) and trials with fear (dotted line). Data are expressed as mean ± SEM, n = 7-8. *p <0.05, **p<0.01, ***p <0.001, LPS versus Saline. ^{a}p <0.05, ^{aa}p<0.01, ^{aaa}p <0.001, main effect of trial type.

Supplementary Table S1. Maternal Immune Activation Model Reporting Guidelines Checklist.

Supplementary Figure 1. Maternal care behaviors following maternal immune activation (MIA) during the mid-lactational period. Total number of (A) pup retrievals, (B) passive nursing bouts, (C) nest building behaviors, and (D) maternal drinking bouts. Maternal care was evaluated in the afternoon and night of postnatal day (P) 10 and the morning of P11. Data are expressed as mean ± SEM, n = 7-8. ^{a}p < 0.05, main effect of time.

Supplementary Figure 2. Nutritional profile of milk following maternal immune activation (MIA) during the mid-lactational period. Maternal milk (A) fat concentration (g/L), (B) energy value (kcal/L), (C) protein concentration (mg/mL), (D) lactose concentration (ng/µL), and IgA concentration (mg/mL). Data are expressed as mean ± SEM, n = 7-8. *p < 0.05, ***p <0.001, LPS versus Saline; ^{a}p < 0.05, main effect of time.

Supplementary Figure 3. Cladogram of milk biomarkers associated with treatment group on P10 (determined by LEfSe). Diameter of the nodes indicates relative abundance of taxa for saline (green) and LPS (red) samples. Placement indicates the classification of taxa, where nodes decrease in rank the closer to the center of the diagram.

Supplementary Figure 4. Taxonomy heatmap demonstrating the top fifty most abundant species identified in samples on P10. Treatment group is indicated by the colored bar at the top of the figure (red = saline, blue = LPS). Each row represents the abundance for each taxon, with the taxonomy ID shown on the right. Each column represents the abundance for each sample.

Supplementary Figure 5. Cladogram of milk biomarkers associated with treatment group on P11 (determined by LEfSe). Diameter of the nodes indicates relative abundance of taxa for saline (green) and LPS (red) samples. Placement indicates the classification of taxa, where nodes decrease in rank the closer to the center of the diagram.

Supplementary Figure 6. Taxonomy heatmap demonstrating the top fifty most abundant species identified in samples on P11. Treatment group is indicated by the colored bar at the top of the figure (red = saline, blue = LPS). Each row represents the abundance for each taxon, with the taxonomy ID shown on the right. Each column represents the abundance for each sample.

Supplementary Figure 7. Adolescent offspring behavior following maternal immune activation (MIA) during the mid-lactational period. (A) Distance traveled (cm), (B) percent time spent in the center, and duration of time (seconds) spent in the (C) center and (D) perimeter of an open field arena. Data are expressed as mean ± SEM, n = 7-8.

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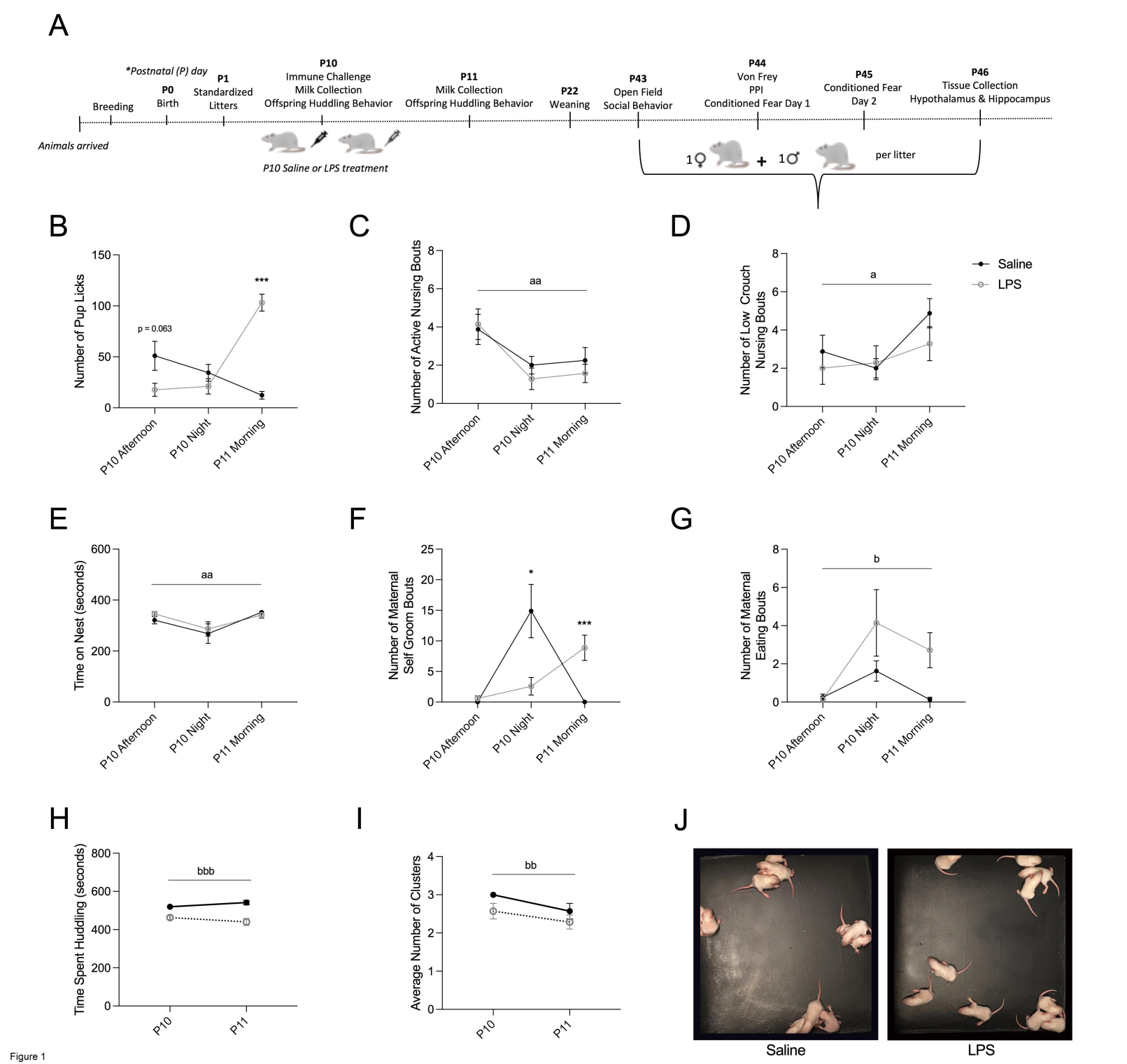
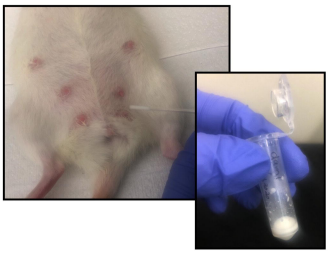
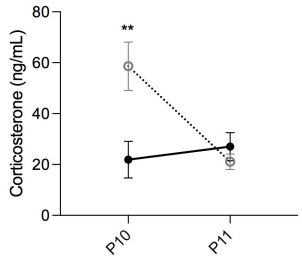
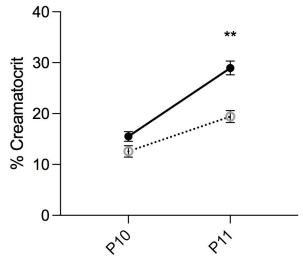
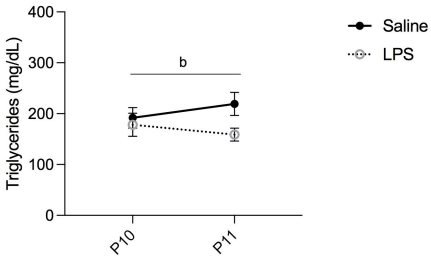

A
*Postnatal (P) day
Breeding
P0
Birth
P1
Standardized
Litters
P10
Immune Challenge
Milk Collection
Offspring Huddling Behavior
P11
Milk Collection
Offspring Huddling Behavior
P22
Weaning
P43
Open Field
Social Behavior
P44
Von Frey
PPI
Conditioned Fear Day 1
P45
Conditioned Fear
Day 2
P46
Tissue Collection
Hypothalamus & Hippocampus
Animals arrived
P10 Saline or LPS treatment
1♀ + 1♂ per litter
B
Number of Pup Licks
p = 0.063
***
P10 Afternoon
P10 Night
P11 Morning
C
Number of Active Nursing Bouts
aa
D
Number of Low Crouch Nursing Bouts
a
Saline
LPS
E
Time on Nest (seconds)
aa
F
Number of Maternal Self Groom Bouts
*
***
G
Number of Maternal Eating Bouts
b
H
Time Spent Huddling (seconds)
bbb
P10
P11
I
Average Number of Clusters
bb
J
Saline
LPS

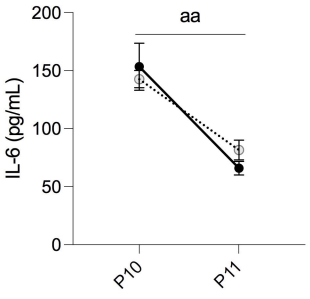
Figure 1

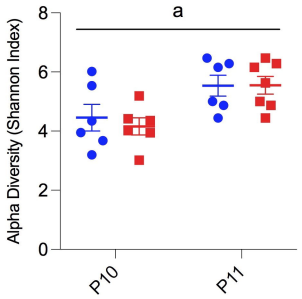


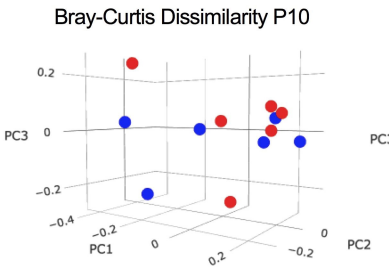

B
Corticosterone (ng/mL)
80
60
40
20
0
**
P10
P11

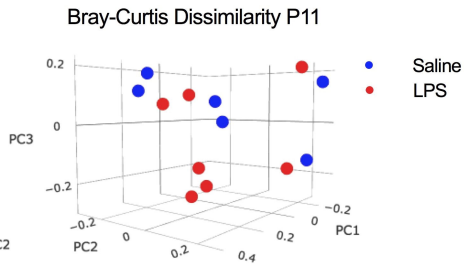

C
% Creamatocrit
40
30
20
10
0
**
P10
P11

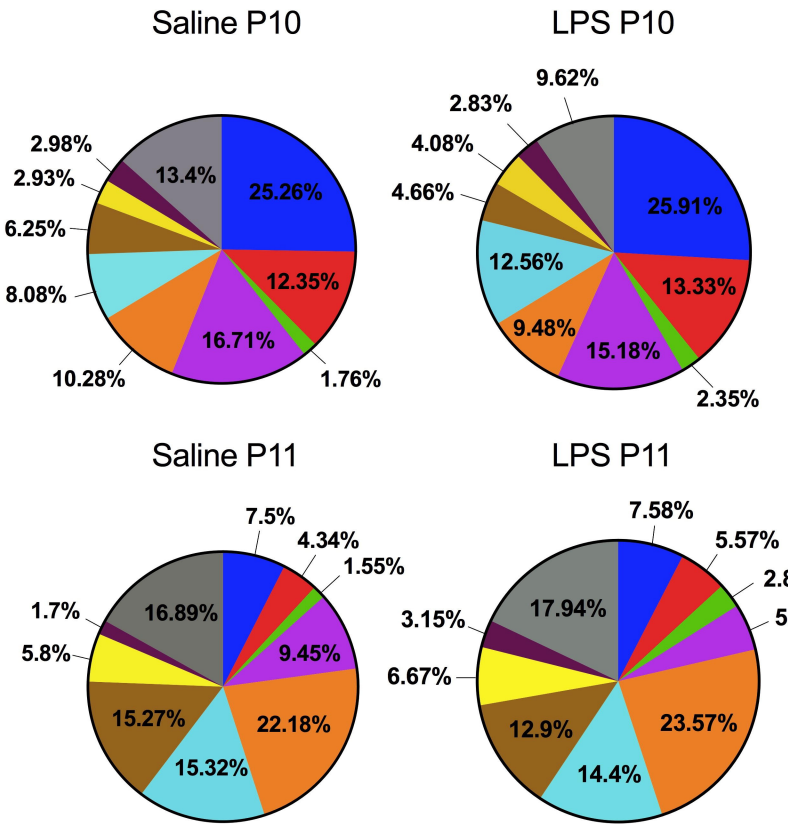

D
Triglycerides (mg/dL)
400
300
200
100
0
b
P10
P11
Saline
LPS

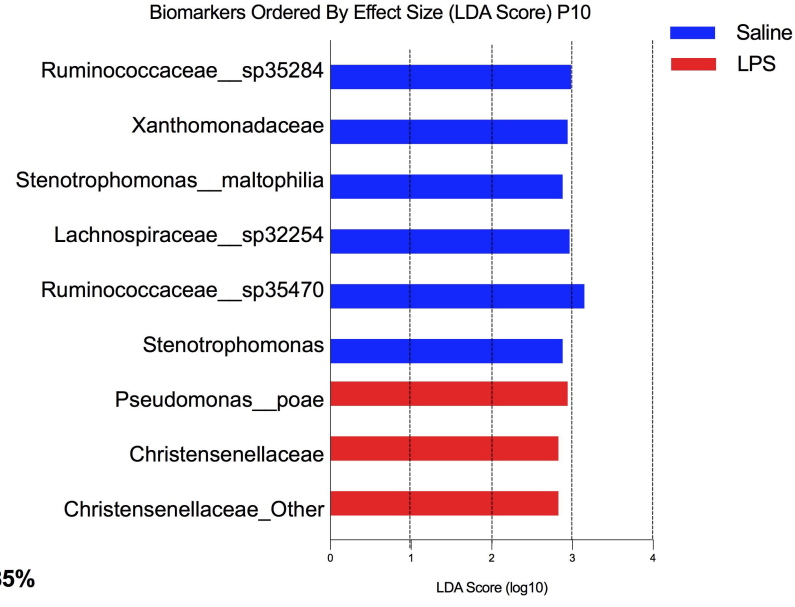

E
IL-6 (pg/mL)
200
150
100
50
0
aa
P10
P11


F
Alpha Diversity (Shannon Index)
8
6
4
2
0
a
P10
P11


G
Bray-Curtis Dissimilarity P10
PC3
PC1
PC2


H
Bray-Curtis Dissimilarity P11
PC3
PC2
PC1
Saline
LPS


I
Saline P10
25.26%
12.35%
1.76%
10.28%
16.71%
8.08%
6.25%
2.93%
2.98%
13.4%
LPS P10
25.91%
13.33%
2.35%
15.18%
9.48%
12.56%
4.66%
4.08%
2.83%
9.62%
Saline P11
22.18%
15.32%
15.27%
5.8%
1.7%
16.89%
9.45%
1.55%
4.34%
7.5%
LPS P11
23.57%
14.4%
12.9%
6.67%
3.15%
17.94%
5.37%
2.85%
5.57%
7.58%
Micrococcaceae
Staphylococcaceae
Lactobacillaceae
Streptococcaceae
Lachnospiraceae
Peptostreptococcaceae
Ruminococcaceae
Erysipelotrichaceae
Pseudomonadaceae
Other


J
Biomarkers Ordered By Effect Size (LDA Score) P10
Ruminococcaceae__sp35284
Xanthomonadaceae
Stenotrophomonas__maltophilia
Lachnospiraceae__sp32254
Ruminococcaceae__sp35470
Stenotrophomonas
Pseudomonas__poae
Christensenellaceae
Christensenellaceae_Other
LDA Score (log10)
Saline
LPS

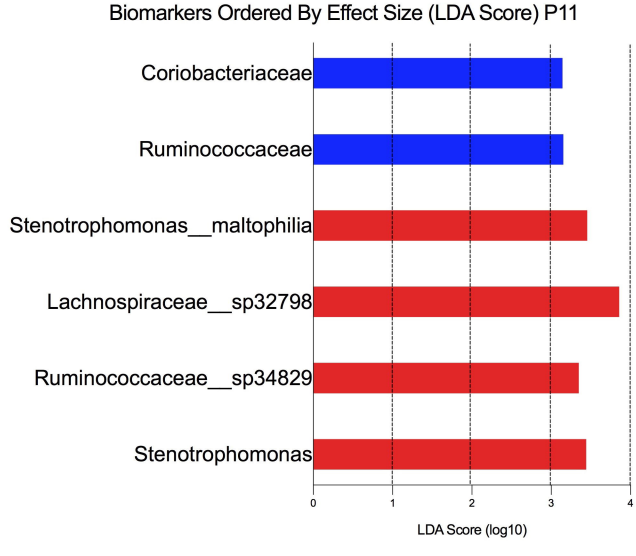

Biomarkers Ordered By Effect Size (LDA Score) P11
Coriobacteriaceae
Ruminococcaceae
Stenotrophomonas__maltophilia
Lachnospiraceae__sp32798
Ruminococcaceae__sp34829
Stenotrophomonas
LDA Score (log10)

Figure 2

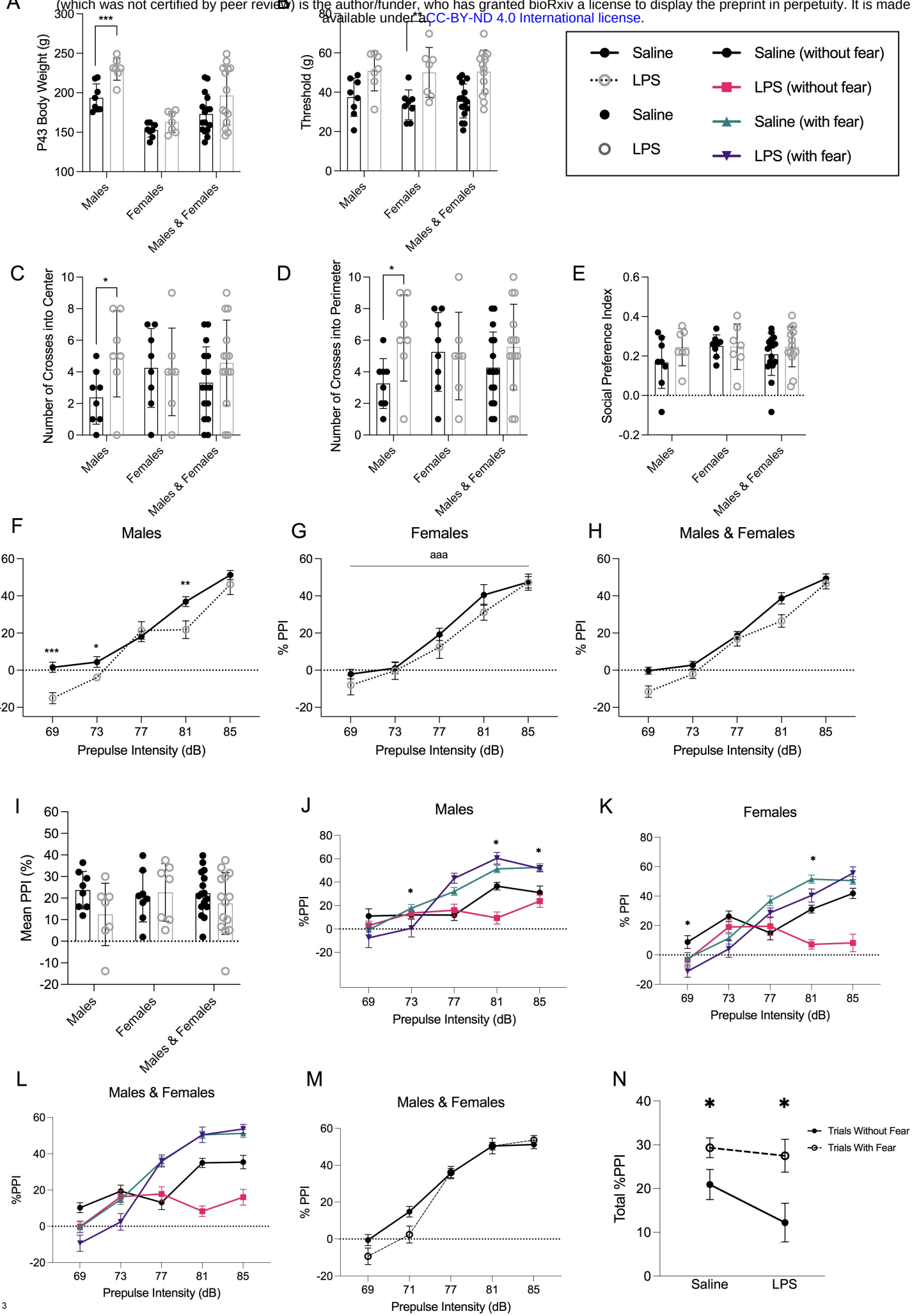


Figure 3