Lasting consequences on physiology and social behavior following cesarean delivery in prairie voles

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ABSTRACT

Cesarean delivery is associated with diminished plasma levels of several 'birth-signaling' hormones, such as oxytocin and vasopressin. These same hormones have been previously shown to exert organizational effects when acting in early life. For example, our previous work found a broadly gregarious phenotype in prairie voles exposed to oxytocin at birth. Meanwhile, cesarean delivery has been previously associated with changes in social behavior and metabolic processes related to oxytocin and vasopressin. In the present study, we investigated the long-term neurodevelopmental consequences of cesarean delivery in prairie voles. After cross-fostering, vole pups delivered either via cesarean or vaginal delivery were studied throughout development. Cesarean-delivered pups responded to isolation differently in terms of their vocalizations, huddled in less cohesive groups under warmed conditions, and shed less heat. As young adults, we observed no differences in anxiety-like or alloparental behavior. However, in adulthood, cesarean-delivered voles of both sexes failed to form partner preferences with opposite sex conspecifics. In a follow-up study, we replicated this deficit in partner-preference formation among cesarean-delivered voles and were able to normalize pair-bonding behavior by treating cesarean-delivered vole pups with oxytocin (0.25 mg/kg) at delivery. We have thus far detected minor differences in regional oxytocin receptor expression within the brains of cesarean-delivered voles. These results speak to the possibility of unintended developmental consequences of a birth mode which currently accounts for 32.9% of deliveries in the U.S. and suggest that further research should be directed at whether hormone replacement at delivery could avert such effects.

INTRODUCTION

In recent decades, the rate of delivery by cesarean section (CS) has risen to 32.9% of all births in the United States [1–3]. Despite its widespread adoption, we have only recently begun to explore the possibility that CS delivery could produce neurodevelopmental consequences [4], spurred on by epidemiological associations between CS delivery and increased risk profiles for: autism spectrum disorders, attention deficit / hyperactivity disorder, and overweight / obesity [3,5–11]. While recent analyses suggest the association between CS and neurodevelopmental disorders arises from unmeasured familiar confounding [12,13], other subclinical outcomes may still occur. Furthermore, purely correlational studies in humans will remain unable to resolve this question definitively, and experimental studies in humans are ethically infeasible. This leaves experimental studies in animal models to address this gap in our knowledge. Whereas humans and rodents differ in terms of cortical development at birth [14], all mammals must navigate a common set of challenges pertaining to the initiation of independent homeostasis upon delivery. It is here that two potential mechanisms for how CS could impact development arises: first, CS may impact development through disturbances of microbial colonization that typically begins upon passage through the birth canal [15,16]. Secondly, CS may affect development through diminished levels of the 'birth-signaling hormones' during a sensitive period in development.

Levels of oxytocin (OXT) [17,18], vasopressin [19,20], glucocorticoids, epinephrine, and norepinephrine all surge during vaginal delivery (VD) [21–24], and the levels of these hormones in the plasma of the neonate are lower after CS delivery compared to those of VD neonates, particularly if CS occurs prior to the onset of labor [25]. Furthermore, CS delivery is often performed relatively early in labor, prior to the recommendations laid out by the American College of Obstetricians and Gynecologists [26], resulting in still lower birth signaling hormone levels [24,27]. The surging levels of the 'birth-signaling hormones' are important because they help accomplish the transition to independent homeostasis that must begin at birth [25,28]. Lower levels of these hormones raises the prospect of developmental programming because the perinatal is a sensitive period in development [25,28–30]. One domain that appears particularly sensitive to early-life OXT manipulation is social behavior [31–34]. Most studies of CS in humans have focused on clinical outcomes, such as autism spectrum disorder, but there have been findings to suggest CS impacts human social behavior as well. A study of ~11,000 children found CS delivery associated with a delay in personal social skills at 9 months [35], and in a study of early childhood, emergency CS was associated with peer problems [36].

In the present study we chose to explore the neurodevelopmental consequences of CS delivery with a focus on social behavior (Experiment 1) and, in a follow-up study (Experiment 2), attempt to normalize development through direct administration of OXT to CS offspring. Both experiments were heavily influenced by our prior work that examined how OXT exposure on the day of delivery influenced neurodevelopment in the prairie vole [34]. We selected a battery of tests aimed at behaviors known to be under the regulation of OXT, including the production of isolation-induced ultrasonic vocalizations (USVs) on postnatal days (PNDs) 1 and 4 [34], behavioral thermoregulation on PND 7 [37], consolation behavior toward a stressed cage-mate on PND 45-46 [38], anxiety-like behavior on PND 48-52 [39], alloparental responsiveness on PND 50-55 [34], and pair-bonding on PND 60-66 [34]. Upon completion of this behavioral battery, subjects were sacrificed and brain tissue collected for autoradiographic analysis of the OXT receptor (OTR) and vasopressin receptor (V1aR) as well as gut tissue and gut contents for histology and microbiome analysis.

METHODS

Subjects: Subjects were laboratory-bred prairie voles (*Microtus ochrogaster*), descendants of a wild-caught stock captured near Champaign, Illinois. The stock was systematically outbred. Breeding pairs were housed in large polycarbonate cages (44cm x 22cm x 16cm) and same sex offspring pairs were housed in smaller polycarbonate cages (27cm x 16cm x 16cm) after weaning on postnatal day (PND) 20. Animals were given food (high-fiber Purina rabbit chow) and water ad libitum, cotton nestlets for nesting material in breeding cages, and were maintained at room temperature (22°) on a 14:10 light:dark cycle. To generate experimental subjects, pairs were created using stud males aged 60-240 days and primiparous adult female voles aged 60-90 days. Pregnant female dams bore litters either via vaginal delivery (VD) or cesarean section (CS). In the CS

condition, surgical delivery occurred via laparotomy following 90 seconds of CO2 anesthesia according to the protocol developed by the Forger lab [40,41], an approach which avoids the confound of pharmacological anesthesia. CS delivery was thus a terminal procedure so as to avoid the confound of surgery affecting maternal behavior and because CS vole dams do not respond maternally to pups [42]. Following delivery (CS) or discovery (VD), litters were cross-fostered to parents that had had litters of their own within the previous 3 days. In Experiment 2, we added a third condition: CS+OXT where CS-delivered pups were immediately injected subcutaneously with 0.25 mg/kg OXT in the form of Pitocin. Likewise, in Experiment 2, CS and VD pups were injected subcutaneously with saline immediately after delivery or discovery. Offspring were then studied throughout development according to the following protocols. For Experiment 1, a total of 23 CS and 23 VD litters were generated, and for experiment 2, 8 CS, 8 VD, and 7 CS+OXT litters were generated. However, not all offspring were used for all measures, owing either to logistic challenges or data loss due to experimenter error. Final sample sizes for each measure are specified below.

Ultrasonic vocalizations (USVs): According to previously published methods [34], we recorded isolation-induced USVs from vole pups on PNDs 1 and 4. Briefly, pups were removed from the nest and tested individually for 5 minutes at room temperature. USVs were recorded using an Avisoft UltraSoundGate 116Hme microphone, sampling at 192 KHz. Vocalizations were detected using Avisoft-SASlab Pro version 5.2.07. A total of 68 CS pups and 88 VD pups had USVs recorded for Experiment 1, and 32 CS, 33 VD pups and 25 CS+OXT pups had USVs recorded for Experiment 2.

Thermography: On PND-7, offspring were tested as litters to characterize thermoregulation in terms of skin surface temperature and behavior. In Experiment 1, a total of 19 CS and 21 VD litters were tested. However, a combination of experimenter error and technical issues prevented us from completing a sufficient number of litters to analyze for Experiment 2. Litters were removed from the nest and placed into a jacketed beaker wherein ambient temperature could be manipulated via passage of heated / chilled water through the walls of the chamber. For 30 minutes litters were exposed to 33° (warm phase), at which point the chamber's water was changed to 22° and once the chamber walls equilibrated to this new temperature (~2-4 minutes), observations resumed for another 30 minutes (cool phase). During observations, measures were collected once per minute in the form of both webcam images and thermographic infrared image captured by means of an ICI 9640-P infrared camera (Infrared Cameras Inc.; ICI; Beaumont, TX). The resulting images were then analyzed using custom Matlab code. For the behavioral measures (webcam), images were binarized and litter huddles analyzed in terms of: 1) the total number of discontinuous clumps and/or lone pups, and 2) the total perimeter of the litter. Thus, a more cohesive litter would have fewer clumps and a shorter perimeter. For the thermographic measures (infrared), images were analyzed in terms of the number of pixels > 30.5°. The parameter of number of pixels > 30.5° was chosen because it best correlated with manually collected intrascapular measures of surface temperature in mouse pups (Harshaw et al., manuscript in preparation). While the walls of the chamber were 33° during the warm phase, the floor of the chamber consisted of a thin foam sheet which remained at < 30°. Thermographic images were analyzed in terms of the number of pixels per image over 30.5° using a repeated measures linear mixed effects model with birth mode (CS vs. VD), time as main effects and accounting for litter weight as a random effect.

Consolation: Between PND-45 and 46, subjects were tested for conciliatory behavior exhibited toward a stressed cage mate. In each pair of animals, one animal was subjected to 30 minutes of restraint stress and the behavior of the remaining animal was then assessed for 30 minutes when the stressed animal was returned to the cage. Behavior was analyzed via two observers blind to experimental condition and also via idTracker. Manually scored conciliatory behaviors included: allogrooming, huddling and sniffing. Automatically scored behaviors included: average distance between the two animals and the time spent within one body length of the stressed animal. The consolation test was only included in Experiment 1, for which a total of 12 CS males, 5 CS females, 8 VD males and 8 VD females were included.

Open Field: Open Field testing was carried out according to previously used methods [34]. Between PND-48 and 52, subjects were tested individually in a plexiglass arena (42 x4 2 cm) for 20 minutes. Behavior was analyzed using idTracker [43] in terms of total distance traveled and time spent in the center of the arena. For Experiment 1, a total of 16 CS males, 7 CS females, 17 VD males and 20 VD females were tested; for

Experiment 2, a total of 13 CS males, 21 CS females, 10 VD males, 20 VD females, 11 CS+OXT males, and 13 CS+OXT females were tested.

Alloparenting: Testing of alloparental responsiveness was carried out according to previously used methods [34]. Between PND-50 and 55, subjects were tested individually in a novel cage for 20 minutes for caregiving behavior directed toward a novel, unrelated pup aged 1-3 days. Measures of interest included time spent: Not in contact with the pup ('Not in Contact'), Sniffing or making incidental contact with the pup ('Contact'), Licking and/or grooming the pup ('Licking / Grooming'), and huddling over the pup ('Huddling'). In Experiment 1, a total of 17 CS males, 9 CS females, 20 VD males and 23 VD females were tested; in Experiment 2, a total of 11 CS males, 18 CS females, 8 VD males, 19 VD females, 9 CS+OXT males, and 13 CS+OXT females were tested.

Pair-bonding and Partner Preference Testing (PPT): Testing of pair-bonding and partner preference formation was carried out according to previously used methods [44]. Between PND-60 and 66, subjects were paired with an opposite sex adult and allowed to cohabit for 24 hours, after which time subjects were tested in the PPT (Figure __). Subjects' were presented with the familiar opposite-sex animal they had cohabited with ('Partner') as well as a novel opposite-sex animal ('Stranger'), each of whom were tethered individual chambers while the subject was able to freely travel. Behavior was analyzed using idTracker [43,44] in terms of the time subjects spent in close proximity to either the Partner or Stranger. We applied a further immobility criterion in addition to proximity so that this measure correlates with the 'side by side contact' measure of the traditional, manually-scored PPT [44]. In Experiment 1, a total of 17 CS males, 11 CS females, 22 VD males, and 23 VD females were tested; in Experiment 2, a total of 4 CS males, 13 CS females, 6 VD males, 9 VD females, 10 CS+OXT males and 10 CS+OXT females were tested.

Sacrifice and brain tissue collection: In Experiment 1, one day after the completion of the PPT, subjects were anesthetized with isoflurane and sacrificed for brain tissue collection. Brains were flash frozen on dry ice and section at 20µm for autoradiography according to previously published methods [34,44]. Briefly, either the OXT receptor (OTR) or vasopressin receptor (V1aR) were visualized using specific radiolabeled ligands and radiosensitive film. For OTR binding [^{125}I]-ornithine vasotocin analog [(^{125}I)OVTA] [vasotocin, d(CH_2)$_5$[Tyr(Me)^2, Thr^4, Orn^8, (^{125}I)Tyr^9-NH_2]; 2200 Ci/mmol] was used (NEN Nuclear, Boston, MA, USA). For V1aR binding ^{125}I-lin-vasopressin [^{125}I-phe- nylacetyl-D-Tyr(ME)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2] (NEN Nuclear) was used. Slides were exposed to Kodak BioMaxMR film (Kodak, Rochester, NY, USA) with ^{125}I microscale standards (American Radiolabeled Chemicals, Inc., St., Louis, MO, USA). Slides were exposed for 168 h for OTR binding and 96 h for V1aR binding. Three coronal slices were measured for each animal at each of three landmarks aimed at capturing the following brain regions: 1) nucleus accumbens, 2) lateral septum and medial preoptic area, and 3) amygdala and hypothalamus. The complete list of brain regions investigated for OTR included the: agranular insular cortex, bed nucleus of the stria terminalis, central amygdala, cingulate cortex, claustrum, entorhinal cortex, lateral septum, nucleus accumbens core and shell, paraventricular nucleus of the hypothalamus, and parietal cortex. The complete list of brain regions investigated for V1aR included the: basolateral amygdala, bed nucleus of the stria terminalis, central amygdala, laterodorsal thalamus, medial cingulate cortex, paraventricular nucleus of the hypothalamus, preoptic area, retrosplenial cortex, and ventral pallidum. A total of 12 CS males, 12 CS females, 12 VD males and 12 VD females were included.

Images of slides were digitally scanned at 1800dpi. Three images of each subject, matched for anterior-posterior position, were then registered to one another using Photoshop (Adobe Systems Inc., San Jose, CA). A measure of non-specific binding (NSB) was also taken for each section from a gray matter region where minimal binding is detected. The NSB value was subtracted from the binding value for each section and a mean was then calculated for each section. Optical density was measured within each atlas-defined ROI using custom designed Matlab code and a slightly modified version of the prairie vole brain atlas we originally developed for magnetic resonance imaging (Yee et al., 2016). Results were calculated as regional averages of optical density (which corresponds to receptor density). Images were then consolidated into group composites for the purpose of visualizing group differences. Heat maps were generated in Matlab for each group-by-group comparison by subtracting one group composite from another.

Sacrifice and gut tissue/stool collection: In Experiment 1, when subjects were anesthetized with isoflurane and sacrificed for brain tissue collection, gut tissue and stool were also collected. Intestinal tissue from the terminal

ileum and proximal colon was rapidly dissected in cold PBS and immediately flash frozen in isopentane/dry ice and stored at -80° Celsius for RNA isolation and qPCR. Stool from the cecum was collected and flash frozen in isopentane/dry ice and stored at -80°C for microbiome analyses.

RNA Extraction, cDNA synthesis, and qPCR: To extract RNA, intestinal tissue was homogenized in Trizol® (Thermo-Fisher Scientific), vortexed for 10 min at 2000 rpm and placed at room temperature (RT) for 15 min. Chloroform was then added to the Trizol solution at a dilution of 1:5 and vortexed for 2 min at 2000 rpm. Tissue samples sat at RT for 3 min, were centrifuged at 11,800 rpm at 4°C for 15 min and the aqueous phase was removed. Isopropanol was added to the aqueous phase at a dilution of 1:1 to precipitate RNA and samples were vortexed again. Samples sat at RT for 10 min, were centrifuged at 11,800 rpm at 4°C for 15 min and RNA pellets were rinsed two times in ice-cold ethanol (75%). Pellets were then resuspended in 20 µl of nuclease-free water and frozen at -80°C until cDNA synthesis. The QuantiTect Reverse Transcription Kit (Quiagen) was used to synthesize cDNA. For each tissue sample, 200ng of RNA in 12 µl nuclease-free H_2O was pre-treated with gDNase at 42°C for 2 min. 8 µl of master (primer-mix plus reverse transcriptase) was then added to each sample. Samples were heated in a thermocycler to 42°C for 30 min followed by 95°C for 3 min. qPCR was run on a Mastercycler ep realplex (Eppendorf) using the SYBR Green PCR Kit (Quiagen). PCR primers for prairie vole genes were designed in the lab using NIH primer blast and NIH nucleotide search, Beacon Designer and Eurofins Oligo analyses tool. Primers were purchased from Integrated DNA technologies. See Table _ for primer sequences. Relative gene expression was calculated using the 2-ΔΔCT method, relative to 18 S (house-keeping gene) and the lowest sample on the plate (Williamson et al., 2011; Livak & Schmittgen, 2001).

Microbiome analysis: Microbiome samples underwent 16S rRNA sequencing to identify bacterial genera/species. Standard protocols from earthmicrobiome.org were used for library preparation. DNeasy Powersoil Kit (Qiagen, Germantown, MD) was used to extract DNA and PCR was performed to amplify the V4 hypervariable region of the 16S rRNA gene with individually barcoded primers (515F-806R; Parada et al., 2016; Apprill et al., 2015; Caporaso et al., 2011, 2012). The PCR product was then purified using PCR Purification Kit (Qiagen). The concentration of DNA was measured using Quant-iT Picogreen Assay (Thermofisher Scientific) and an equimolar pool was made of all samples. The pool was sequenced at the MGH Next-Generation Sequencing Core using an Illumina MiSeq Instrument that performed 20,000 paired-end 250bp reads per sample (Illumina, San Diego, CA, USA).

The sequenced raw fastq files are processed with QIIME software package (v. 2018.2.0) [#1]. The sequences with low quality score (on average less than 25) are truncated to 240bp and by using deblur algorithm with default settings the erroneous sequence reads are filtered [#2]. The remaining high quality sequences are aligned with mafft plugin [#3]. Next, the aligned sequences are masked to remove highly variable positions and a phylogenetic tree is generated from the masked alignment by FastTree plugin [#4]. Alpha and beta diversity metrics and Principal Component Analysis plots are generated by default QIIME2 plugins [#1,#5,#6]. To assign taxonomies to our sequences we have used QIIME2's feature-classifier plugin and pre-trained Naïve Bayes classifier, which has been trained on the Greengenes 13_8 99% operational taxonomic units OTUs. Differential abundance analysis of OTUs are performed by ANCOM [#7]. The Kruskal-Wallis test is used assess the statistical significance between groups. Benjamini-Hochberg false discovery rate (FDR) was employed for multiple testing corrections [#12]. The FDR threshold level was set at ___. The extent of differences in the taxonomic profiles was substantiated further by analyzing LDA effect sizes calculated through LEfSe (LDA >4, $P < .05$).

Analysis: For measures involving repeated measures (USVs, thermography) results were analyzed using the lmer() function from the *lme4* library (__) followed by calling the anova() function on the resulting model. Otherwise, results were analyzed using ANOVA. In both cases, sex and birth mode were considered as main effects. For the pup measures of USV production and themography, a repeated measures linear mixed effects model with time (age in the case of USVs, minutes of test in the case of thermography) and birth mode (CS vs. VD) as main effects and accounting for weight and litter as random effects. Autoradiographic analyses of OTR and V1aR densities were compared on a regional basis, first by bilaterally averaging each subject's measures, then using a repeated measures linear mixed effects model, so as to accommodate the repeated sampling of each brain region (two slices, often with bilateral representation). Outlying autoradiographic data points were

detected and flagged automatically using Grubb's test and then manually confirmed. Post-hoc analyses were carried out using Tukey's HSD. When results were not normally distributed, data were analyzed by Kruskal-Wallis and Mann-Whitney tests.

RESULTS

Weights, Experiment 1: VD and CS litters did not differ in litter size; VD litters had 4.52 ± 0.28 pups, and CS litters had 4.52 ± 0.25 pups. At delivery/discovery, VD pups weighed more than CS pups ($p = 0.001$); VD pups weighed 2.81 ± 0.08g, and CS pups weighed 2.41 ± 0.07g. On PND-1 and PND-4, there was a trend towards CS pups weighing less than VD pups($F(1,30.216) = 2.76$, $p = 0.1$). On PND-1, VD pups weighed 3.15 ± 0.04g, and CS pups weighed 2.90 ± 0.04g; on PND-4, VD pups weighed 4.37 ± 0.07g, and CD pups weighed 4.23 ± 0.07g. On PND-7, there was no difference in litter weights ($p = 0.35$); VD litters weighed 25.38 ± 1.2g and CS litters weighed 23.72 ± 1.19g.

Weights, Experiment 2: Again, there was no significant difference in litter size; VD litters had 5 ± 0.7 pups, CS litters had 4.38 ± 0.4 pups, and CS+OXT litters had 3.71 ± 0.5 pups. At delivery/discovery, there was a significant effect of birth mode on pup weight ($F(2,20) = 9.071$, $p = 0.002$), with CS pups weighing less than pups of either other group ($p < 0.004$ for both comparisons); VD pups weighed 2.85 ± 0.11g, CS pups weighed 2.39 ± 0.08g, and CS+OXT pups weighed 2.88 ± 0.09g. On PND-1 there was a significant main effect of birth mode on pup weight, with both VD and CS+OXT pups weighing more than CS pups ($p = 0.019$ and $p = 0.014$ respectively). However, by PND-4, there was no significant difference between groups' pup weights. We were not able to make conclusions about litter weights on PND-7 due to small sample sizes.

USVs, Experiment 1: Across PND-1 and PND-4, 288 recordings were made from pups of 43 litters (n = 22 VD, n = 21 CS). In analyzing the total number of USVs, there were significant main effects of birth mode, age, and weight, along with a birth mode by age interaction effect ($F(1,230) = 4.35$, $p = 0.038$), which post hoc analysis revealed as VD pups producing significantly fewer USVs on PND-4 compared to PND-1 ($p = 0.002$) and CS pups producing more USVs than VD pups on PND-4 ($p = 0.018$). CS pups showed no age-related decline in USVs ($p = 0.643$).

USVs, Experiment 2: Across PND-1 and PND-4, 172 recordings were made from pups of 23 litters (n = 8 VD, n = 8 CS, n = 7 CS+OXT). In analyzing the total number of USVs, there was a birth mode by age interaction effect ($F(2,154.54) = 3.67$, $p = 0.028$), which post hoc analysis revealed as a trend towards more VD pups producing more USVs on PND-4 compared to CS pups ($p = 0.061$).

Thermography, Experiment 1: Litters of both birth mode conditions experienced a decline in surface warmth throughout testing, but CS litters consistently emitted less warmth (Figure __). There was an expected main effect of time, as well as a main effect of birth mode and an interaction between time and birth mode ($F(5,175) = 2.38$, $p = 0.04$). Post hoc analyses revealed CS litters to have fewer pixels > 30.5° during the three initial warm phases of testing (minutes 0-30, $p < 0.025$ for all comparisons). For huddling behavior, there were also main effects of both time and birth mode, along with a time by birth mode interaction ($F(5,146.63) = 2.88$, $p = 0.016$), which post hoc analysis revealed as CS litters having significantly more disparate huddles during the first 10 minutes of testing ($p < 0.001$) and a trend towards the same in the second 10 minutes ($p = 0.058$). For the total perimeter of a huddled litter, there were main effects of litter weight, time, and birth mode ($F(1,135.46) = 26.07$, $p < 0.001$), which post hoc analysis revealed as CS litters displaying higher total perimeters of their huddles during the first twenty minutes ($p < 0.041$ for phases 1, 2, 4 and 6) and a trend for minutes 40-49 ($p = 0.067$).

Consolation, Experiment 1: There was no effect of either sex or birth mode on any consolation behavior under observation ($p > 0.05$ for all comparisons). Anecdotally, animals did appear to display the expected set of conciliatory behaviors directed at their stressed cage-mates.

Open Field, Experiment 1: There was no effect of neither sex nor birth mode on time in the center of the OFT ($p > 0.05$ for both comparisons). However, there was a sex by birth mode interaction on total distance traveled ($F(1,56) = 7.096$, $p = 0.01$), which post hoc analysis revealed as a trend towards greater locomotion in CS females ($p = 0.086$) and a trend towards less locomotion in CS males ($p = 0.055$).

Open Field, Experiment 2: Again, there was no effect of neither sex nor birth mode on time in the center of the OFT ($p > 0.05$ for both comparisons). There was however, a main effect of sex ($F(2,79) = 4.096$, $p = 0.046$), with greater locomotion in males, and a trend toward an effect of birth mode on total distance traveled, ($p = 0.057$), such that the two CS conditions, when collapsed, moved significantly more than VD counterparts ($p = 0.016$).

Alloparenting, Experiments 1 and 2: Female prairie voles attack novel pups at high rates. In Experiment 1, females attacked pups at a higher rate than males (chi-squared = 6.42, $p = 0.011$). Although we observed no treatment nor sex by treatment interactions in the proportion of animals attacking the pup, this still left us with a small sample size of female CS animals that displayed alloparental behavior (only 4 out of 9 CS females did not attack the pup). Because of this, the alloparenting results for Experiments 1 and 2 were combined into a single analysis. There was no effect of birth mode on any alloparental behavior under observation (Figure __, $p > 0.05$ for all comparisons). Females were generally less alloparental than males, displaying for instance greater time not in contact with the pup ($F(1,103) = 12.762$, $p < 0.001$) and less time huddling the pup ($F(1,103) = 8.907$, $p = 0.004$). These same patterns persisted when we analyzed Experiments 1 and 2 separately (data not shown).

Partner Preference, Experiment 1: Both male and female VD animals formed partner preferences as defined as spending significantly more time in close social contact with the Partner vs. the Stranger ($p < 0.05$ for both comparisons). Neither male nor female CS animals formed partner preferences, although female CS animals showed a trend toward spending more time in close social contact with the Partner vs. the Stranger ($p = 0.099$).

Partner Preference, Experiment 2: Again, both male and female VD animals formed partner preferences ($p < 0.05$ for both comparisons) and neither male nor female CS animals formed partner preferences ($p > 0.05$ for both comparisons). Both male and female CS+OXT animals formed partner preferences ($p < 0.05$ for both comparisons).

CD has sex-specific effects on the gut microbiome and intestinal epithelium

Microbiome, Experiment 1: We used 16S ribosomal RNA sequencing to assess the microbial composition of the cecal microbiome at PND 60 in male and female prairie vole offspring born via VD or CD and cross-fostered to surrogate dams at birth. An individual's microbial community structure can be assessed by alpha diversity which includes measures of richness (the number of different bacterial species present) and evenness (whether all species have a similar abundance within the community). We did not observe any significant differences in richness or evenness for males (observed OTUs: $p=0.73$; Fig. _A; Pielou's evenness: $p=0.76$; Fig._B) or females (observed OTUs: $p=0.22$; Fig. _C; Pielou's evenness: $p=0.51$; Fig._D) based on birth mode. Beta diversity refers to a difference in microbial community structure between individuals. Principle Coordinate Analysis (PCoA) of beta diversity indices revealed a trend for the distinct clustering of CD and VD microbiome profiles for male (Jaccard: $p=0.056$; Bray-Curtis dissimilarity: $p=0.068$: Fig. _E) but not female offspring (Jaccard: $p=0.123$; Bray-Curtis dissimilarity: $p<0.607$; Fig._F). LEFSE analyses showed….

Gut Gene expression, Experiment 1:

Autoradiography, Experiment 1: Overall, there were few differences in either OTR or V1aR observed. In the agranular insular cortex, we noted a main effect of birth mode on OTR density ($F(1,46) = 4.375$, $p = 0.042$), with CS animals having greater OTR. In the claustrum, there was a main effect of birth mode on OTR density ($F(1,48) = 6.827$, $p = 0.012$), with CS animals having greater OTR. However, in no other brain region did we note an effect of birth mode, and therefore, considering the number of comparisons made, we cannot place much confidence in these two differences.

In the central amygdala, we observed a sex by birth mode interaction on OTR ($F(1,46) = 5.573$, $p = 0.0225$) such that CS females had greater OTR than VD females ($p = 0.023$). Across the lateral septum at 2 anterior-posterior positions, there were consistent main effects of sex on OTR ($F(1,48) = 5.111$, $p = 0.028$ and $F(1,48) = 4.63$, $p = 0.036$), with females having greater OTR. In the cortical amygdala, we noted a main effect of sex on V1aR ($F(1,45) = 4.616$, $p = 0.037$), with males having greater V1aR density. In the ventral pallidum, there was a main effect of sex ($F(1,45) = 4.051$, $p = 0.05$), such that females had greater V1aR density, as well as a sex by birth mode interaction ($F(1,45) = 6.6107$, $p = 0.014$), however no post-hoc comparisons of relevance were significant.

DISCUSSION

In this study we observed a set of behavioral differences across development arising from CS delivery, only some of which were replicated in a follow-up experiment. Briefly, in Experiment 1, CS pups were found to produce more USVs on PND-4, had lower skin temperature and huddled less cohesively on PND-7, failed to form partner preferences as adults, and showed minor changes in OTR density in the agranular insular cortex and claustrum.

[insert one sentence summary of microbiome / gut histology findings here]

However, of these findings, we are most confident in the impaired pair-bonding phenotype because we were able to replicate that effect in Experiment 2. CS pups in Experiment 2 tended to show the opposite pattern in terms of USVs, with CS pups producing fewer calls, and unfortunately, we were not able to complete thermographic analysis at PND-7, nor autoradiographic or metabolomic analyses upon sacrifice. The most noteworthy finding of the present study is therefore that voles delivered via CS fail to form partner preferences, an effect which can be prevented by treating CS pups with 0.25 mg/kg OXT immediately after CS delivery.

Previous work in mice has found that CS pups either produce more calls on PND-9 [45–47] or produce an equivalent number of calls to VD counterparts [40,48] or USVs with decreased amplitudes [40], however, these studies were all in mice at a later stage of development than the current work. Our prior study of VD vole pups on PNDs 1 and 4 found that exposure to prenatal OXT led to increased USV production [34]. This led us to hypothesize that CS would diminish calls, which was not reliably observed in the present study. In the same prior study, we also observed that prenatal OXT exposure led to a broadly gregarious phenotype in adult offspring. OXT-exposed voles displayed increased alloparental responsiveness (in two separate experiments) and increased time in close social contact with opposite-sex adults (in the only experiment that addressed this behavior). Importantly, prenatal OXT exposure did not affect the selectivity aspect of pair-bonding in that prior work, i.e. voles were no more nor less monogamous, whereas in the present study, CS impacted the selectivity of pair-bonding such that CS animals failed to form selective partner preferences. We are unaware of any work that has considered pair-bonding or romantic attachments in humans as a function of birth mode.

Direct treatment of CS pups with 0.25 mg/kg OXT immediately after delivery was able to prevent the deficit in pair-bonding observed in CS offspring. This is in line with our hypothesis that CS can affect neurodevelopment by impacting levels of the birth-signaling hormones during the sensitive period around birth. This also matches recently published work which found that daily OXT treatment (~0.1 or 1 mg/kg) between

days 1 and 5 could rescue social behavior deficits in mice delivered by CS [48]. In that study, CS pups did not experience different levels of parental care -an important consideration that we hope to pursue in the future. While we observed no effect of CS on anxiety-like behavior in the OFT in the present study, the recent study by Morais and colleagues found that CS led to diminished anxiety-like (rather “repetitive”?) behavior in offspring mice, which could be prevent by OXT treatment [48]. CS mice also showed less preference for their natal nest, which was restored by treatment with the ~1 mg/kg OXT dose. Finally, CS mice showed deficits in social novelty preference, which were restored by the ~0.1 mg/kg OXT dose.

One important alternative explanation to the present findings which must be considered is that offspring in the CS condition were affected by early delivery and low birth weight. In Experiments 1 and 2 CS pups weighed less than VD pups, though some of this discrepancy must be due to CS pups being freshly delivered whereas VD pups were discovered, typically within 12 hours of birth. At delivery, CS pups weighed ~2.4g while VD pups weighed ~2.8g. Some of this difference in weight must be attributed to VD pups having 0-12 hours of nursing prior to discovery. However, we cannot completely discount the possible role for early delivery on the developmental outcomes observed here. A meta-analysis of 4.4 million people found that adults who were born preterm or of low birth weight were less likely to ever experience a romantic relationship, sexual intercourse, or parenthood than peers born full-term (odds ratios of 0.72, 0.43, and 0.77 respectively) [49]. There are clearly many differences between pre-term and CS delivery, but this does establish that birth experience can shape social bonding in adulthood. Furthermore, OXT is not the only birth-signaling hormone affected by CS. In rats, CS delivery influences the regulation of dopamine in later life via diminished levels of the birth signaling hormone epinephrine [50–52], which would also affect social attachment as dopamine is critical to pair-bond formation and maintenance in prairie voles [53]. It stands to reason that treatment with vasopressin, corticosterone, epinephrine or norepinephrine could each also normalize development in CS offspring.

Similar to prior work examining the paraventricular nucleus of the hypothalamus in mice [48], we did not find evidence for substantial changes in OXTR and V1aR in the adult brains of CS offspring. Although we observed CS animals having greater OTR in the agranular insular cortex and claustrum, as well as CS females having greater OTR in the central amygdala, we must consider the large number of comparisons made. For the time being, we must consider these findings as promising directions for future work to either confirm or reject. One intriguing possibility comes from recent work indicating that ligand expression patterns rather than those of the receptors may be impacted by CS delivery [54]. Ramlall and colleagues recently reported that CS delivery led to fewer and smaller vasopressin cells and smaller OXT cells in the paraventricular nucleus of adult mice [54]. Diminished vasopressin or OXT signaling could certainly lead to deficits in pair-bonding in prairie voles [55].

Previous work in humans [56] and lambs [57,58] has found that CS impacts thermoregulation in offspring, which we take as evidence that the initiation of independent homeostasis has been compromised. The present findings extend this effect substantially further into development than previous work, which focused on the first postnatal day. Here, we observed lower surface temperatures in CS pups along with less cohesive huddling behavior. It remains for future work to examine how these differences relate; that is, are they independent or does less huddling lead to lower surface temperatures (or vice versa)? OXT is a potent thermoregulatory hormone, one which directly activates brown adipose tissue [37,59], thus by diminishing OXT levels during the sensitive period around delivery, CS could impact the onset of independent thermoregulation. Differences in thermoregulation would also contribute to the production of isolation-induced USVs, as temperature is arguably the largest driving factor for USV production [60]. However, if a thermoregulatory difference persisted into adulthood, we would also have expected this to have impacted alloparenting, which we did not observe here. In any case, changes in thermoregulation should be considered a possible avenue for how CS impacts development alongside neuroendocrine and microbial mechanisms. One major advantage of working with prairie voles is that they are adapted to cool climates and appear to avoid the chronic cold stress that mice and rats face in conventional ‘room temperature’ (22°) housing [61]. This is especially important when considering the developmental consequences of cold stress on vulnerable pups, where prairie voles have the added benefit of biparental care providing additional warmth in the nest. Thus, we view the prairie vole as an especially well-suited model for studying the neurodevelopmental consequences of CS delivery.

CS is worthy of further investigation given its epidemiological associations with various later life health outcomes and also simply due to its prevalence. Regardless of whether CS delivery is causally related to immune or metabolic or social differences, CS affects the signaling of several important hormones during a sensitive period in development, which raises the prospect of enduring consequences. These consequences may or may not rise to the level of a clinical diagnosis, but deserve further investigation regardless. Given that CS rates are rising around the world and stand already at nearly a third of all births in the U.S., we owe public health a fuller accounting of the benefits and drawbacks that CS delivery affords future generations.

FIGURE LEGENDS

Figure 1

Production of ultrasonic vocalizations (USVs) by vole pups delivered either via cesarean section (CS) or vaginal delivery (VD). In Experiment 1 (panel A), CS pups produced significantly more USVs on postnatal day (PND) 4 (* denotes $p = 0.018$, n = 288 total recordings from 43 litters (n = 22 VD, n = 21 CS)). However, in Experiment 2 (panel B), CS pups tended to produce fewer USVs than VD pups on PND-4 († denotes $p = 0.061$, n = 172 total recordings from 23 litters (n = 8 VD, n = 8 CS, n = 7 CS+OXT)).

Figure 2

Thermoregulatory behavior in CS and VD vole litters on PND-7. Nineteen CS and 21 VD litters were tested for 30 minutes at 33° followed by 30 minutes at 22°. A representative thermographic image is shown in panel A. CS litters had less warm surface temperatures compared to VD litters during the warm phase (panel B, * denotes $p < 0.025$ for all comparisons). A representative image is shown in panel C having undergone automated identification of separate blobs (note that the seemingly disconnected hindleg in blue would have been excluded for having been too small). CS litters' huddles were less cohesive throughout the majority of the testing session, during both the warm and cool phases (panel D, * denotes $p < 0.041$, († denotes $p = 0.067$).

Figure 3

Partner preference formation in CS and VD voles on PND 60-68. The partner preference test (PPT) paradigm is shown in panel A. In Experiment 1, both female (F) and male (M) VD voles showed significant preferences for the Partner over the Stranger in terms of time spent in close social contact, as expected given prairie voles' well-established social monogamy (panel B, * denotes $p < 0.05$ for both comparisons). CS animals showed no such preference, though there was a trend for females to spend more time with the Partner over the Stranger († denotes $p = 0.099$). Results from Experiment 1 include data from 17 CS males, 11 CS females, 22 VD males, and 23 VD females. In Experiment 2, VD voles again showed a significant partner preference, as did CS+OXT voles (panel C, * denotes $p < 0.05$ for all comparisons), whereas once again, CS animals showed no such preference. Results from Experiment include data from 4 CS males, 13 CS females, 6 VD males, 9 VD females, 10 CS+OXT males and 10 CS+OXT females were tested.

Figure 4

There were no differences in adult microbiome composition between the CS and VD conditions across either sex (A-D, $p > 0.05$ for all comparisons). In a principal component analysis of microbial diversity, there was a trend toward a difference between CS and VD males ($p = 0.068$, E) but no such difference among females (F).

Figure 5

There were two level 4 differences between male CS and VD voles where CS males had greater abundance (top) and there was a single level 6 difference where VD males had greater abundance (bottom).

Figure 6

Gut sample western blot analyses for the small intestine (A) and large intestine (B). While there were no birth mode differences among females, CS males showed decreased levels of OCLN, TJP, S100B, and GFAP ($p < 0.05$ for all comparisons).

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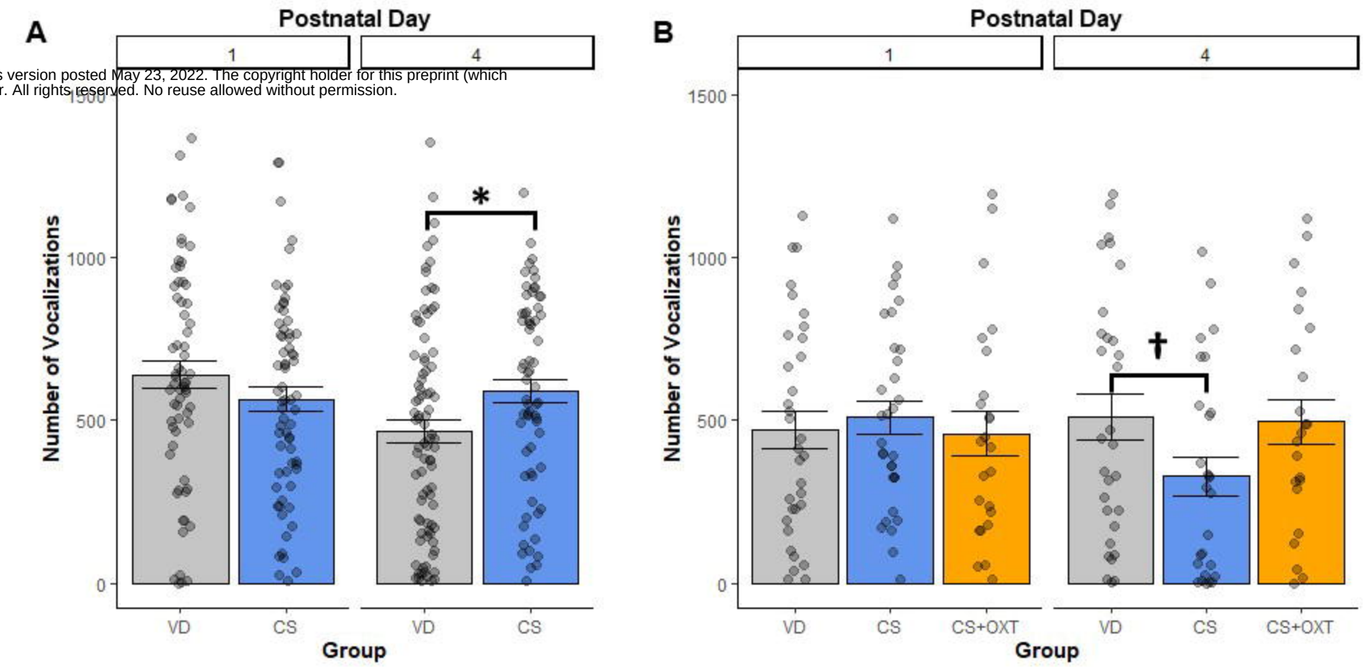

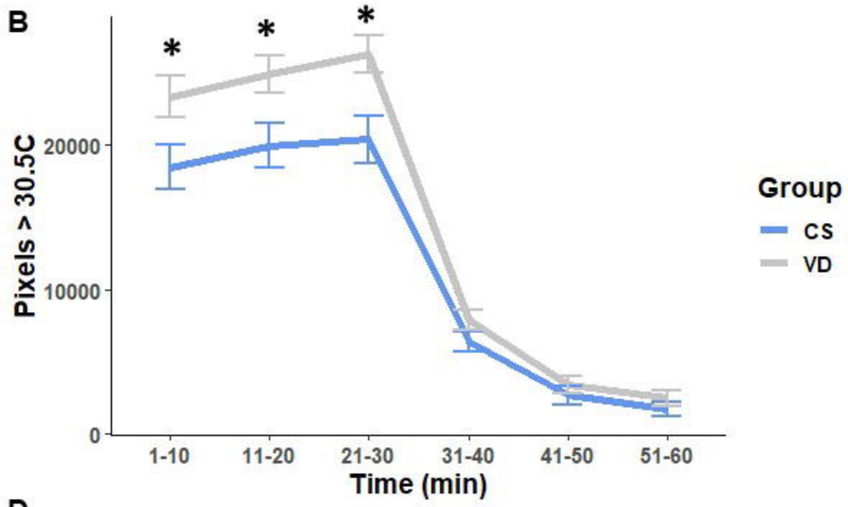

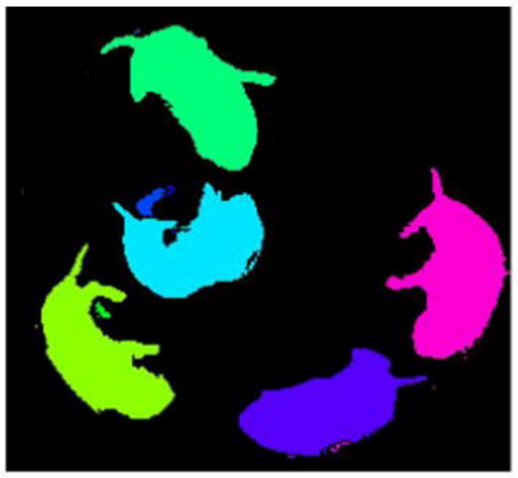

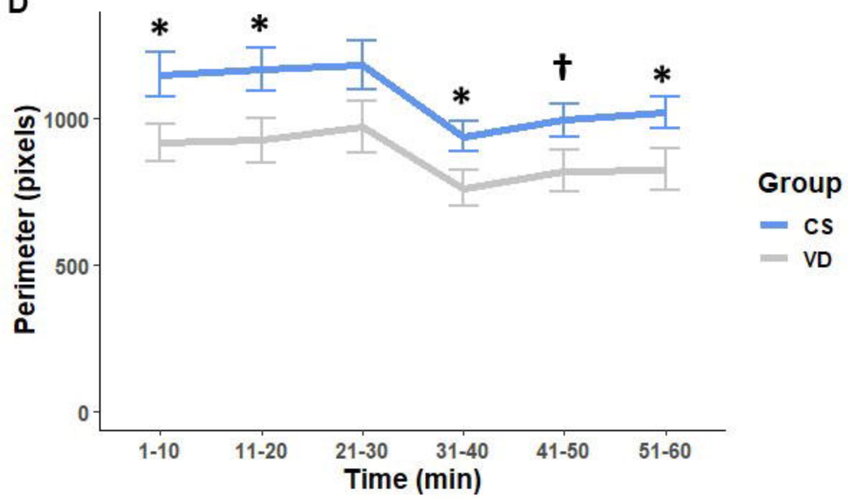

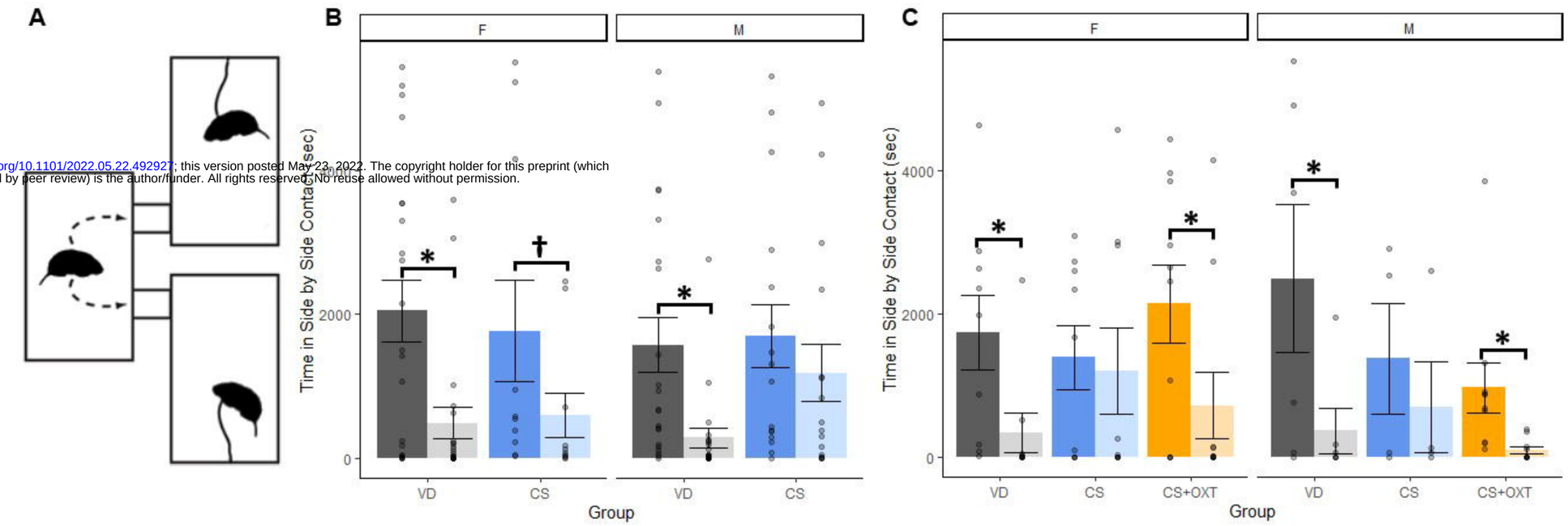

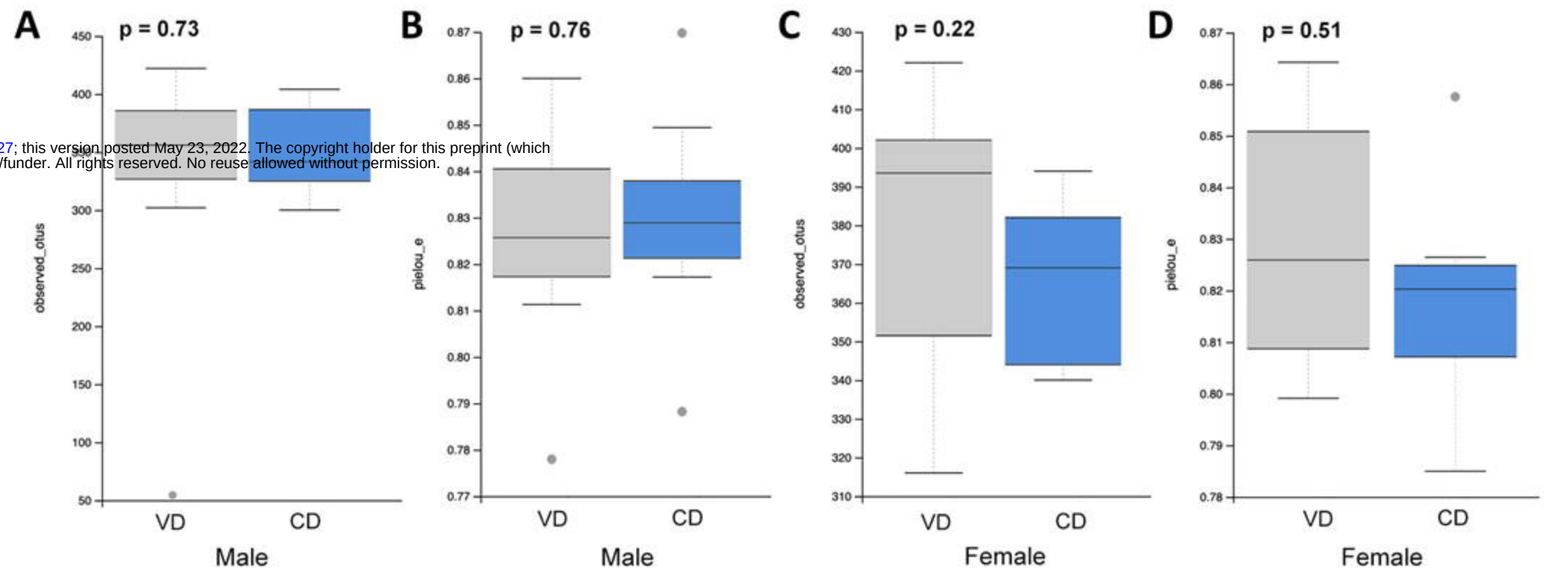

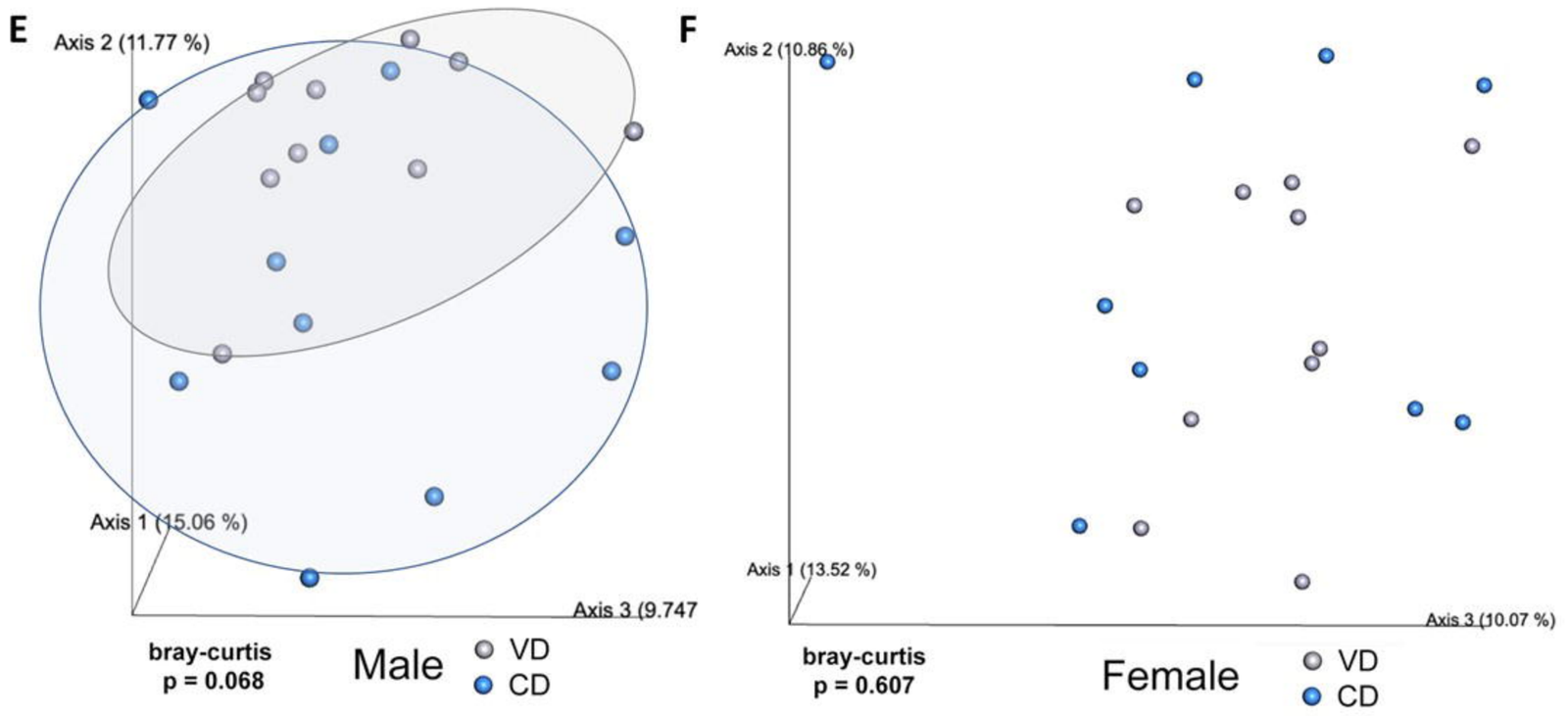

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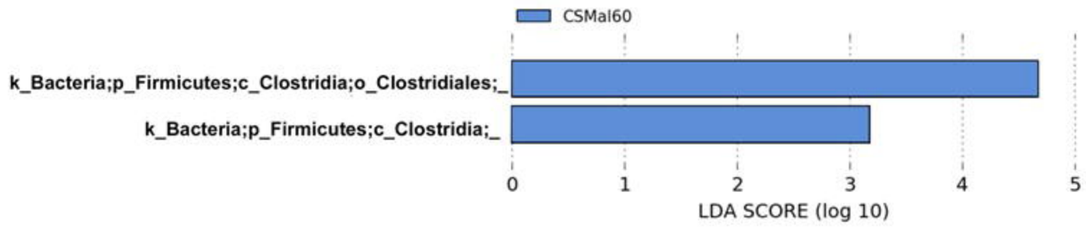

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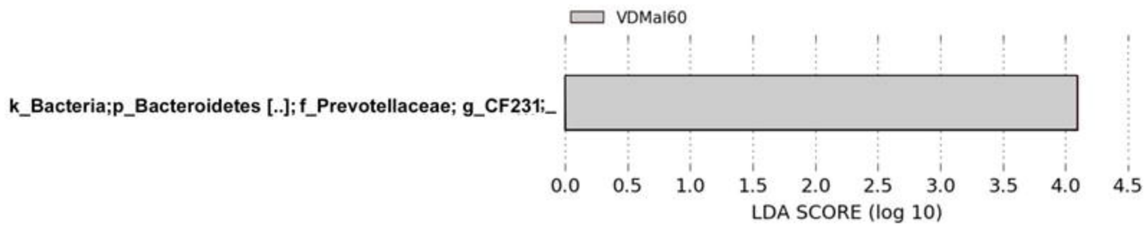

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