A deep learning pipeline (MAS-PRT) reveals that prenatal CBD, but not THC, uniquely drives weight-dependent hyper-reactivity in maternal pup retrieval

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Abstract

This study investigates the impact of prenatal cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC) exposure on maternal caregiving in C57BL/6J mice, addressing the limitations of manual scoring by introducing the Machine-Automated Scoring of the Pup Retrieval Test (MAS-PRT). This fully automated deep learning pipeline integrates Multi-Animal DeepLabCut for dam and pup tracking with Detectron2 for dynamic nest reconstruction. Validated against 170 manual annotation trials, MAS-PRT achieved high concordance (< 6% deviation), enabling the extraction of granular metrics like path length and encounter distance without experimenter bias.

While gestational exposure (GD5–GD18) did not impair general nest-building, pups from both groups exhibited reduced body weight at postnatal day 5. Crucially, the study demonstrates that automated behavioral scoring can detect subtle maternal effects missed by traditional averaging methods. Although standard latency analyses suggested no impairment, Cox proportional hazards modeling revealed a distinct divergence: CBD-exposed dams showed a heightened, weight-dependent probability of retrieving their lightest pups. This paradoxical enhancement in maternal reactivity was absent in THC-exposed dams, indicating that CBD and THC produce distinct maternal behavioral profiles despite similar offspring weight outcomes.

Rather than indicating a unilateral deficit, the data suggest that prenatal CBD exposure reshapes the mother-offspring dyad through a convergence of altered offspring distress signaling and modulated maternal sensitivity. Ultimately, MAS-PRT proves to be a robust, scalable tool for behavioral neuroscience. Its ability to uncover nuanced phenotypes obscured by conventional methods provides the precision necessary for defining complex behavioral endophenotypes, establishing a scalable methodology for future neurodevelopmental research on parenting.

Introduction

Prenatal exposure to cannabis poses significant risks to both maternal well-being and fetal development [1,2]. As the most commonly used illicit substance among pregnant and breastfeeding women [2–5], cannabis compounds—specifically cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC)—readily cross the placental barrier due to their lipophilic nature, accumulating in fetal tissues [6,7]. While THC is the primary psychoactive component, recent years have seen a surge in the use of CBD, a non-intoxicating derivative of *Cannabis sativa*, by expectant mothers seeking relief from pregnancy-related discomforts such as anxiety, nausea, and insomnia[7–9]. Despite this growing trend, systematic research regarding the specific effects of CBD on maternal care remains scarce [1,10,11].

Maternal care is a critical determinant of offspring health outcomes. In humans, substance use during pregnancy is frequently associated with atypical parenting behaviors that disrupt mother-infant attachment [12,13]. Neurobiologically, the transition to motherhood involves profound remodeling of regulatory circuits, emotional processing, and reward systems[14]. These processes overlap significantly

with those underlying drug addiction[15], suggesting that prenatal cannabinoid exposure could interfere with the neural mechanisms governing parental behavior [14]. Poor maternal care in rodents has been directly linked to neurodevelopmental deficits and neuropsychiatric vulnerabilities in offspring [16], highlighting the necessity of examining parent-infant interactions in the context of drug exposure.

Existing preclinical literature on cannabinoid effects on maternal behavior presents a complex picture. Studies involving low-to-moderate doses of THC (2–5 mg/kg) have generally reported no significant alterations in maternal dedication, nest building, or reproductive parameters [17–19]. Conversely, emerging evidence suggests that while prenatal CBD exposure may not immediately abolish maternal behaviors, it can influence offspring outcomes, such as protecting against OCD-like behaviors when offspring are cross-fostered to drug-free mothers[10]. However, these studies often rely on manual scoring methods that may lack the sensitivity to detect subtle, trial-level variations in maternal reactivity or fail to account for confounding variables like pup weight and sex.

Given the intense dyadic interaction between dams and pups during the early postnatal period, maternal behavior serves as a primary environmental cue shaping the offspring's developmental trajectory [14,20] (REF). To address the limitations of current research, this study pursued two primary objectives. First, we investigated the impact of prenatal CBD and THC exposure on a comprehensive range of maternal caregiving behaviors, encompassing both pup-directed actions (nursing, pup retrieval) and non-pup-directed behaviors (nest building). Second, to overcome the constraints of manual observation, we developed and validated a novel, fully automated deep learning pipeline: the Machine-Automated Scoring of the Pup Retrieval Test (MAS-PRT). By integrating dynamic nest detection with high-precision tracking of dams and pups, this tool enables a granular analysis of maternal reactivity. Using this approach, we demonstrate that while overall daily home-cage behavior remains largely intact, prenatal CBD exposure specifically alters the mother-offspring dyad by modifying reactivity to pup distress cues, a nuance that traditional averaging methods may dismiss.

Materials and Methods

Animals and Housing: All experimental procedures adhered to the European Communities Council Directive (86/609/EEC) and the United States NIH Guide for the Care and Use of Laboratory Animals. The project was authorized by the French Ethical Committee (APAFIS#49376). Adult male and female C57BL/6J mice (6–8 weeks old) were purchased from Charles River. Animals were housed in standard lid-topped Plexiglas cages (42 × 27 × 14 cm) under controlled conditions: temperature 21 ± 1°C, relative humidity 60 ± 10%, and a 12-hour light/dark cycle (lights on at 07:00). Food and water were available *ad libitum*.

Following a one-week acclimation period, females were paired with a single male in the late afternoon. The presence of a vaginal plug was designated as gestational day 0 (GD0). Pregnant dams were then individually housed. Postnatal day 0 (PND0) was defined as the day of birth. Litters were not culled at

birth to preserve natural litter size variability. Pups were weaned on PND21 and subsequently housed separately by sex.

Prenatal Drug Exposure: From GD5 to GD18, pregnant dams received daily subcutaneous (s.c.) injections of either a vehicle solution, 3 mg/kg of CBD, or 3 mg/kg of THC[6,21,22]. The cannabinoids were obtained from the NIDA Drug Supply Program. Both compounds were dissolved separately in a vehicle solution consisting of Cremophor-EL (Sigma-Aldrich), ethanol, and saline in a 1:1:18 ratio, administered at a volume of 4 mL/kg. Control dams ("Sham") received an equivalent volume of the vehicle solution alone.

Nest Building Skills: Nest quality was assessed daily at 09:30. Dams were provided with 11 g of pressed aspen wood wool from the onset of gestation. Nests were manually scored using a modified version of Deacon's 5-point scale[23,24], ranging from 0 to 4[25]:

- 0: No shredding; no visible nest site.
- 1: Material shredded (completely or partially); identifiable but flat nest.
- 2: Material shredded; saucer-shaped nest formed.
- 3: Material completely shredded; walls tall enough to cover the animal.
- 4: Material completely shredded; fully enclosed nest with a complete roof covering the animal.

Home-Cage Dam Behavior: Maternal behavior was observed daily from PND1 to PND10 at 10:00 for 20 minutes. During each session, the dam and litter were observed for five 60-second intervals (total observation time: 300 seconds/day/litter). Observations focused on:

1. Nest Occupancy: Whether the dam was inside or outside the nest. Time spent outside (eating, drinking, grooming) was noted but not quantified.
2. Nursing Behavior: Time spent inside the nest, classified as:

- *Active Nursing*: Dam arched over pups with rigid limbs and head depressed.
- *Passive Nursing*: Dam lying flat on the litter with minimal limb support.

Pup Retrieval Test: Maternal reactivity was assessed on PND5, following a protocol adapted from Winters et al. (2022). Testing occurred between 10:30 and 13:00 in a homemade and soundproofed plastic arena equipped with a top-mounted camera (Foscam C2 IP camera; 2046 × 2046 resolution, 15 fps) positioned 50 cm above the floor.

The procedure for each pup in a litter was as follows:

1. The home cage was moved to the testing room, and the dam was allowed to acclimate for 45 minutes with the nest undisturbed and the lid of the cage open.
2. One pup was removed, weighed, sexed[26], marked, and placed in a heated glass beaker (35°C).

3. Once the dam was confirmed to be in the nest, the isolated pup was placed in the corner of the cage furthest from the nest.
4. The dam had 90 seconds to retrieve the pup. If retrieval did not occur, the pup was returned to the nest, and the next littermate was tested. Trials where the dam failed to retrieve the pup within 90 seconds were classified as censored events.
5. Metrics Recorded:

- *Time to First Encounter (tFE)*: Time from start until the dam contacted the pup.
- *Time to Retrieve (tR)*: Total time from start until the pup was returned to the nest.
- *Success*: Binary outcome (1 = retrieved within 90s; 0 = not retrieved).

Automated Analysis Pipeline (MAS-PRT) Videos were processed using a custom deep learning pipeline, the Machine-Automated Scoring of the Pup Retrieval Test (MAS-PRT). The workflow involved three stages: (1) tracking dam and pup movements, (2) automatic nest detection, and (3) data extraction. Videos were pre-processed using the open-source video transcoder HandBrake (cropped to the home cage, resized to 720p, and converted to grayscale).

The model and instructions for adapting it to specific experimental setups are available online on GitHub: https://mas.readthedocs.io/en/latest/index.html

1. Tracking and pose estimation: We utilized Multi-Animal DeepLabCut (DLC) [27]to track positions. The model was trained on 988 frames (selected via k-means clustering) using a ResNet50 backbone. Training continued for 40,000 iterations until performance plateaued. The dataset was split 95% for training and 5% for validation. Seven key points were labeled for the dam and five for each pup (see Supplementary Fig. 1A–B).
2. Nest detection: Nest localization was performed using Detectron2[28]. The model was trained on 66 annotated frames from 33 videos. During analysis, 20 pseudo-randomly selected frames per video were used to generate a polygonal representation of the nest. An average polygon was computed per video to account for dynamic changes in nest shape. Validation tests confirmed that 20 frames provided accuracy comparable to a 100-frame benchmark (Supplementary Fig. 2).
3. Data extraction and validation: The pipeline integrated DLC and Detectron2 outputs to extract metrics including: PRT success, tR, tFE, distance to first encounter (dFE), distance from first encounter to retrieval (dFE-R), total distance moved (dT), and nest area (cm^2). The system was validated against manual annotations from 170 trials, demonstrating high concordance with minimal deviation in latency metrics (Supplementary Fig. 3).

Statistical Analysis were performed using GraphPad Prism (v11).

- Gestational Parameters: Analyzed using the Kruskal-Wallis test.
- Nest Quality and Home-Cage Behavior: Analyzed using linear mixed-effects models with Geisser-Greenhouse correction. Treatment and Time were fixed effects; subject was a random effect to

account for repeated measures. Tukey's post-hoc test was applied for multiple comparisons.

- Pup Retrieval Test:
    - Averaged dam performance per litter: Two-way ANOVA followed by Tukey's post hoc multiple comparison test or Kruskal-Wallis test.
    - Single pup analysis: Due to the right-censored nature of the data (maximum 90s limit), we employed time-to-event (survival) analysis. A Cox proportional hazards regression (Cox-PH) model was fitted to predict first encounter and retrieval latency. Covariates included pup sex, trial order, treatment, pup weight, and the interaction term (pup weight × treatment). Dams were clustered to account for within-litter correlations.
- Variance check: Levene's test (SciPy, Python) was used to verify equality of variances in pup weights across trial orders (Supplementary Table 1).
- Significance: All tests were two-tailed, and significance was set at $p < 0.05$.

Results

Prenatal cannabinoid exposure does not modify gestational outcomes.

The endocannabinoid (eCB) system plays a critical role in reproductive physiology, regulating processes such as fertilization, implantation, and neurodevelopment[29,30]. Consequently, exposure to cannabis, its primary phytocannabinoids (THC, CBD), or synthetic agonists has been linked to adverse reproductive outcomes, including impaired fertility[30], reduced ovarian reserve[31], and placental dysfunction leading to restricted fetal growth[30,32,33]. Disruption of eCB signaling can further compromise key events such as embryo transport[34] and uterine implantation[35]. Notably, high-dose oral CBD administration during gestation has previously been associated with reduced pup survival from birth to weaning[10].

In contrast to these reported deleterious effects, our findings indicate that prenatal exposure to CBD or THC at the doses and regimens employed in this study did not significantly impact maternal gestational weight gain, litter size, or the sex ratio at birth (Fig. 1). Furthermore, all litters achieved 100% survival through the weaning period (data not shown), suggesting that the specific dosing protocol used here did not induce overt maternal or fetal toxicity.

Nest-building behavior remains intact following prenatal cannabinoid exposure.

In the wild, pregnant females construct complex nests to ensure offspring survival by providing essential protection and thermal insulation[36–38].

Our results demonstrate that prenatal exposure to CBD or THC did not impair this innate behavior. Dams in all treatment groups successfully constructed nests of high complexity, consistently achieving scores of 3–4. Throughout the first 10 days of the postnatal period, these dams built fully enclosed nests that adequately covered their litters, ensuring necessary protection (Fig. 2A; Supplementary Fig. 4).

Concurrent with nest assessments, daily home-cage observations (20 minutes/day) revealed that dams across all groups predominantly remained in the nest during daylight hours. A single exception was observed in the THC-exposed group on postnatal day 6, which exhibited a transient reduction in nest occupancy compared to controls (Fig. 2B). Overall, these findings indicate that the fundamental drive and ability to construct protective nests and maintain nest occupancy are preserved despite gestational cannabinoid exposure.

Prenatal cannabinoid exposure preserves high nest occupancy and normal nursing patterns.

Pup-directed behaviors, such as nursing and licking, occur primarily within the nest, where the dam's presence is essential for pup protection and healthy development [20,38,39]). Nursing is particularly critical for offspring survival, providing both nutrition and thermoregulation[39,40].

In our study, dams across all treatment groups spent more than half of their time in the nest (Fig. 2B), with most of this time dedicated to nursing (Fig. 3A). Over the first 10 postnatal days, females consistently displayed a strong preference for nursing behaviors (Fig. 3A). While generally stable, we observed a transient reduction in nursing among THC-exposed dams on postnatal day 6, which normalized by day 7.

Furthermore, the profile of nursing behaviors shifted naturally over time. During the first postnatal week, dams primarily engaged in active nursing (Fig. 3B; Supplementary Fig. 5A). By postnatal day 10, passive nursing had become the dominant behavior (Fig. 3C; Supplementary Fig. 5B). These results indicate that prenatal exposure to CBD or THC does not disrupt the overall pattern or intensity of nursing, aside from a brief, self-correcting fluctuation in the THC group.

Prenatal CBD and THC exposure reduces body weight at postnatal day 5 in pups independent of sex.

Cannabis use during pregnancy has been associated with adverse outcomes, including fetal growth restriction and low birth weight[32,41]. Specifically, perinatal exposure to CBD has been shown to influence the metabolic health of offspring[10,42]. Consistent with these findings, our study revealed that pups exposed to CBD or THC during gestation exhibited significantly lower body weights at postnatal day 5 compared to controls (Fig. 4A). This growth deficit was observed consistently in both male and female pups (Fig. 4B), indicating that the impact of prenatal cannabinoid exposure on early growth is not sex specific. Notably, these body weight differences resolved by adulthood (Table 1), suggesting a transient effect on early postnatal growth trajectories.

Table 1

Body weight follow-up from early postnatal period till adulthood. Summary of body weight statistics (median, max, min, n) for females and males across treatments (Sham, CBD, THC) from PND 5 to PND 90. Statistical comparisons via Mixed-effects model (REML) for longitudinal trends and Tukey's multiple comparisons test for pairwise treatment differences at each time point. *P*-values < 0.05 indicate significant differences from Sham controls.

Body weight	Treatment	Median	Max	Min	N	Mixed-effects model (REML)	Tukey´s multiple comparisons test (p-value)
FEMALES							
PND 5	SHAM	3.10	3.80	2.20	34	**PND**	
	CBD	2.50	3.10	1.80	46	F (3,833, 269,6) = 5123; P = < 0,0001	**< 0,0001**
	THC	2.30	3.20	1.80	29	**Treatment** F (2, 127) = 15,05; P = < 0,0001 **Interaction** F (7,666, 269,6) = 1,159; P = 0,3249	**< 0,0001**
PND 10	SHAM	5.75	7.10	4.10	18		
	CBD	4.90	5.90	4.00	27		**0.0068**
	THC	4.70	5.80	3.30	34		**0.0015**
PND 15	SHAM	7.00	7.90	5.70	17		
	CBD	6.40	8.20	5.00	33		**0.2345**
	THC	6.40	7.70	5.30	21		**0.779**
PND 20	SHAM	10.15	11.20	8.50	20		
	CBD	9.65	11.50	7.60	18		**0.0482**
	THC	8.70	11.40	7.10	21		0.4134
PND 30	SHAM	16.30	17.90	13.40	37		
	CBD	15.05	20.40	11.00	28		**< 0,0001**
	THC	14.60	16.40	13.00	19		0.5485
PND 60	SHAM	20.90	22.30	16.80	37		
	CBD	20.35	21.90	17.80	28		0.46
	THC	20.20	22.10	18.80	15		0.9538
PND 90	SHAM	23.24	26.80	19.59	49		
	CBD	22.45	26.20	20.00	23		0.2349
	THC	22.20	26.50	20.20	16		0.9995
MALES							

Body weight	Treatment	Median	Max	Min	N	Mixed-effects model (REML)	Tukey´s multiple comparisons test (p-value)
FEMALES							
PND 5	SHAM	2.90	3.50	2.10	48	**PND**	
	CBD	2.50	3.30	2.00	54	F (3,151, 214,8) = 4563; P = < 0,0001	**< 0,0001**
	THC	2.50	3.10	1.90	35	**Treatment** F (2, 134) = 21,34; P = < 0,0001	**< 0,0001**
PND 10	SHAM	5.70	7.00	4.80	21	**Interaction** F (6,302, 214,8) = 4,245; P = 0,3249	
	CBD	5.00	6.30	3.40	16		**0.0062**
	THC	4.65	5.50	4.20	18		**< 0,0001**
PND 15	SHAM	7.55	9.00	5.20	20		
	CBD	6.20	8.20	5.20	21		**0.0329**
	THC	4.65	5.50	4.20	28		**< 0,0001**
PND 20	SHAM	9.40	11.50	6.90	19		
	CBD	10.50	12.30	9.20	18		**0.047**
	THC	8.00	10.90	5.87	24		**0.0027**
PND 30	SHAM	18.75	21.10	14.00	46		
	CBD	18.00	21.40	8.20	39		**0.0283**
	THC	16.55	18.60	10.50	12		**0.0161**
PND 60	SHAM	27.15	29.90	22.00	46		
	CBD	26.20	30.10	23.20	31		0.1265
	THC	25.50	27.93	23.30	12		**0.0285**
PND 90	SHAM	31.10	36.10	28.10	30		
	CBD	30.15	33.80	26.70	30		0.8223
	THC	31.20	32.70	27.20	16		0.6604

Prenatal cannabinoid exposure does not impair averaged-per-litter maternal performance in the PRT.

Seven days after the cessation of subcutaneous injections (vehicle, CBD, or THC), corresponding to postnatal day 5, dams underwent the pup retrieval test (PRT). The PRT is the most widely used assay for evaluating maternal care in fundamental and preclinical rodent studies[43] and is frequently employed to

study the impact of pharmacological and environmental interventions on maternal behavior[44,45]. In its standard form, the test measures the dam's retrieval response to pup displacement from the nest, a task requiring a coordinated sequence of pup-directed sensorimotor behaviors elicited by the integration of multimodal pup stimuli.

Typical PRT protocols rely on manual scoring, which is prone to spatial and temporal inaccuracies. To address this limitation, we developed an automated detection system to extract PRT data. Both manual and automated analyses demonstrated that averaged per-litter dam performance was not altered by prenatal cannabinoid exposure. Neither CBD- nor THC-exposed dams showed significant differences in latency to approach a displaced pup (Fig. 5A) or in the time taken to complete a successful retrieval (Fig. 5B) compared to controls. Dams consistently performed the task across all pups in the litter, successfully retrieving approximately 75% of the offspring within the allotted time (Fig. 5C).

Discrepancies between manual and automated annotations were minimal, showing only a 1.5% difference in approach latency and a 5.6% difference in retrieval latency (Supplementary Fig. 3). These minor variations did not affect the overall outcomes, confirming the reliability of the automated detection and data extraction.

Maternal locomotor patterns remain unaltered following prenatal cannabinoid exposure.

The effects of acute cannabinoid exposure on locomotion remain complex; while CBD effects are often inconclusive, but predominately producing hypolocomotion[46], THC effects are dose-dependent, with low doses inducing hyperactivity and high doses causing hypoactivity[47]. However, by the time the PRT was conducted, no residual metabolites were detectable in the dams' systems[48] .

Using our automated annotation pipeline, we quantified maternal locomotor behavior by measuring the total distance traveled. Our analysis revealed that dams exposed to CBD or THC exhibited normal movement patterns. This was evidenced by the distance covered to reach the pup (Fig. 6A), the distance traveled between the first encounter and retrieval (Fig. 6B), and the total distance required to complete a successful retrieval (Fig. 6C). These findings indicate that neither prenatal CBD nor THC exposure influences maternal locomotor activity during pup retrieval tasks.

Time-to-event analysis reveals enhanced encounter and retrieval probability of light-weight CBD-exposed pups.

Pup retrieval is a widely used measure of parental responsiveness in rodents, primarily driven by auditory and olfactory cues[25,43,49,50]. Although pups possess a non-functional auditory system at birth, they emit ultrasonic vocalizations (USVs) when isolated, which prompt dams to approach and retrieve them[49]. Previous studies using the same prenatal CBD exposure protocol reported sex-specific differences in offspring outcomes, including USV patterns[21]. Since pup calls are a primary motivator for retrieval[49,51], sex-related variations in vocalizations could influence maternal behavior. Additionally, litter size may impact retrieval performance, as larger litters require more attempts and are associated with smaller pup

body weights[52]. USVs emission serves as a proxy for pups affective state, and it is also linked to pup thermoregulation and body weight[53].

To analyze the latency data, we employed Cox proportional hazards models. This approach appropriately accounts for the right-censored nature of the data (unretrieved pups by the end of the test) and incorporates covariates such as pup order, sex, body weight, and treatment that may influence pup encounter and retrieval likelihood. This robust analysis revealed that CBD-exposed pups exhibited a significantly higher probability of being encountered by their mothers, while THC-exposed pups were spared of this higher reactivity (Fig. 7A, Table 2). The increased likelihood of being encountered applied only to CBD pups weighing less than 2.4g at PND 5; as body weight increased, the probability of encounter for CBD pups decreased (Fig. 7B and Table 2). On the other hand, even though prenatal THC treatment also reduces offspring bodyweight, this treatment did not change the dam's performance in the PRT (Fig. 7C and Table 2).

Table 2

Cox Proportional Hazards regression analysis of latency to encounter the displaced pup. Results derived from multivariate Cox regression model. Hazard ratio (HR) indicates the relative risk of the event occurring compared to the reference level for each covariate. The time-to-event analysis highlights that CBD treatment increases the likelihood of first encounter, but this effect is strictly weight-dependent: only lightweight CBD pups have a higher chance of being encountered. Additionally, increasing trial numbers augment the chances of the event occurring (learning effect). Statistical significance set at p-value < 0.05.

VARIABLE	HR	95% CI	p-value
Trial number	1.096	1.031 to 1.163	**0.0032**
Treatment (CBD)	86.09	4.793 to 1542	**0.0025**
Treatment (THC)	0.5507	0.03214 to 9.812	0.6829
Sex (male)	1.243	0.9308 to 1.667	0.1408
Body weight	1.191	0.6335 to 2.248	0.5872
Treatment (CBD) * body weight	0.1608	0.05464 to 0.4734	**0.0009**
Treatment (THC) * body weight	1.234	0.4173 to 3.554	0.7003

The same analysis was performed on the latency to fully retrieve the pups, thereby completing the task (Fig. 7D, Table 3). Again, lightweight CBD pups showed a reduced failure rate for retrieval (Fig. 7E). The treatment effect was weight-dependent, while prenatal THC did not modify dams' skills to recover the pups (Fig. 7F).

Table 3

Cox Proportional Hazards regression analysis of successful pup retrieval. Results derived from multivariate Cox regression model. Hazard ratio (HR) indicates the relative risk of the event occurring compared to the reference level for each covariate. The time-to-event analysis highlights that CBD treatment increases the likelihood of retrieval, but this effect is strictly weight-dependent: only lightweight CBD pups have a higher chance of being retrieved. The probability of the event is positively associated with both trial frequency and male biological sex. Statistical significance set at *p*-value < 0.05.

VARIABLE	HR	95% CI	p-value
Trial number	1.123	1.056 to 1.193	**0.0002**
Treatment (CBD)	143.1	7.492 to 2753	**0.001**
Treatment (THC)	0.4277	0.02265 to 8.391	0.5738
Sex (male)	1.379	1.025 to 1.865	**0.0338**
Body weight	1.233	0.6353 to 2.403	0.5365
Treatment (CBD) * body weight	0.1351	0.04478 to 0.4064	**0.0004**
Treatment (THC) * body weight	1.421	0.4670 to 4.215	0.5322

Relying solely on average dam retrieval performance (e.g., Fig. 5) would overlook these critical trial-level variations and censoring effects.

Beyond treatment effects, the testing order significantly influenced maternal performance. Pups presented in later trials were more likely to be encountered and retrieved, serving as a proxy for maternal learning effects (Tables 2 and 3). Plus, male sex pups also are more likely to be successfully retrieved. To ensure retrieval order was not biased by pup size, Levene's tests for homogeneity of variance were performed. No significant differences in body weight variance were found across trial numbers, neither in the general population ($p = 0.798$) nor when analyzed within specific treatment groups (Sham $p = 0.981$; CBD $p = 0.596$; THC = 0.994), confirming that pup selection was random with respect to weight.

Discussion

This study utilized a novel deep learning pipeline, MAS-PRT, to investigate the impact of prenatal CBD and THC exposure on maternal care in mice, unmasking a previously hidden endophenotype: a weight-dependent hyper-reactivity in CBD-exposed dams. Unlike controls or THC-exposed dams, mothers prenatally exposed to CBD displayed a significantly higher probability of retrieving their lightest pups, a phenotype that remained invisible to standard mean-latency analyses. This finding underscores a critical limitation of conventional averaging methods, which facilitate "phenotypic masking" by diluting biologically relevant, trial-level variance. While both CBD and THC exposure resulted in transient pup growth restriction that normalized by adulthood, only CBD exposure fundamentally recalibrates the

mother-offspring dyad, suggesting a specific neurobiological or communicative divergence between the two cannabinoids.

The specificity of the CBD effect, observed despite similar pup weight reductions in the THC group, argues against a simple response to low birth weight and instead supports a dyadic interaction model. We propose that prenatal CBD exposure likely alters both the output of the offspring, such as ultrasonic vocalization patterns previously documented in our lab[21], and the sensory-motor integration of the dam. The endocannabinoid system is a modulates maternal circuits in the medial preoptic area and ventral tegmental area[54]. We hypothesize that prenatal CBD exposure may sensitize these pathways, essentially lowering the threshold for the salience of specific distress cues. It remains to be determined whether such maladaptive recalibration of maternal vigilance (characterized by hyper-responsiveness to signals from vulnerable offspring) is paralleled by clinical observations of increased maternal anxiety and hyper-vigilance following gestational cannabis use.

The absence of this hyper-reactivity in THC dams, despite their pups sharing similar physiological stress, implies that CBD uniquely reshapes this communication loop. This challenges the binary view of "impaired versus normal" maternal care, suggesting instead a maladaptive recalibration of maternal vigilance where the dam becomes hyper-responsive to the specific signals emitted by vulnerable offspring.

The discovery of this phenotype highlights the transformative potential of the MAS-PRT methodology. By integrating Multi-Animal DeepLabCut with dynamic nest detection, our pipeline enables a granular, time-to-event analysis using Cox proportional hazards (Cox-PH) models. This statistical framework is particularly well-suited for translational research as it accounts for censored data and integrates several covariates, such as pup weight and trial order, that are typically lost in classical ANOVA-based approaches. While important foundational work[10] provided a baseline by reporting correct PRT performance, the reliance on a protocol that displaces all the litter at once and measures the total latency to recover the first pup and then the full litter may have inadvertently masked the trial-level and weight dependent variance revealed here. Furthermore, the fully automated, batch-processable nature of MAS-PRT provides a scalable solution for high-throughput behavioral phenotyping, removing the subjectivity and 'temporal coarseness' characteristic of manual scoring.

Our approach explicitly modeled the weight-by-treatment interaction, revealing a phenotype that was previously invisible to traditional statistical frameworks. Furthermore, unlike Simba-based approaches like BAMBI[55] that often require manual cage delimitation, MAS-PRT offers a fully automated, batch-processable workflow. This scalability is essential for detecting subtle behavioral shifts in large-scale preclinical studies, moving the field away from subjective manual scoring toward reproducible, high-throughput behavioral phenotyping.

In the context of physiological outcomes, our results align with human and animal literature indicating that prenatal cannabinoid exposure induces transient fetal growth restriction[30,32,33]. While dams

maintained stable weight gain and intact nest-building skills, the reduced pup weight at postnatal day 5, which normalized by day 60, exposes offspring to the metabolic risks associated with rapid "catch-up" growth[41,56].

The dissociation between robust maternal motivation, reflected in stable nursing and nesting patterns, and altered retrieval latencies suggests that CBD specifically targets the neural set-points governing responses to offspring distress rather than inducing a generalized state of maternal neglect. Consequently, the hyper-reactivity identified in the pup retrieval test likely represents a targeted shift in the maternal processing of acute challenges rather than a broader deficit in maternal drive. This distinction is vital; it indicates that while the fundamental maternal motivation remains intact, the sensory-motor integration of distress cues, or the physiological characteristics of the cues themselves, has been significantly recalibrated.

Despite these advances, several limitations remain. A primary constraint is the inability to simultaneously record USVs during the retrieval task. While we hypothesize that altered pup signaling drives the maternal response, future studies should correlate acoustic signatures with retrieval performance to isolate the contributions of pup signaling from maternal sensitivity. Additionally, our automated tracking model is optimized for top-view recordings of C57BL/6 mice; generalization to other strains or recording angles will require retraining. Additionally, while we observed a learning curve consistent with primiparous dams[55], further investigation into the cognitive flexibility of exposed dams will be required to determine if this hyper-reactivity stems from altered executive control or heightened emotional valence.

In conclusion, this study demonstrates that prenatal CBD exposure reshapes the fundamental dynamics of maternal care, driving a paradoxical, weight-dependent hyper-reactivity. By establishing MAS-PRT as a robust, bias-free tool, we provide the field with a framework for uncovering nuanced psychiatric endophenotypes that may have previously been "lost in the average". This work offers a more precise assessment of the neurodevelopmental risks associated with CBD, often perceived as a "safe" cannabinoid, highlighting its capacity to uniquely disrupt the reciprocal bonds of the maternal-offspring dyad.

Declarations

Author Contributions: A.C.-R.: conceptualization, data curation, formal analysis, validation, writing (review and editing). B.J.: conceptualization, software, analysis. J.F.: software. J.P: software, analysis. D.I.: data curation. P.C.: conceptualization, methodology, project administration, supervision. O.J.J.M.: conceptualization, supervision, funding acquisition, methodology, project administration, writing (original draft, review, and editing). All authors have read and agreed to the published version of the manuscript.

Declarations of interest: The authors declare no competing interests.

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Figures

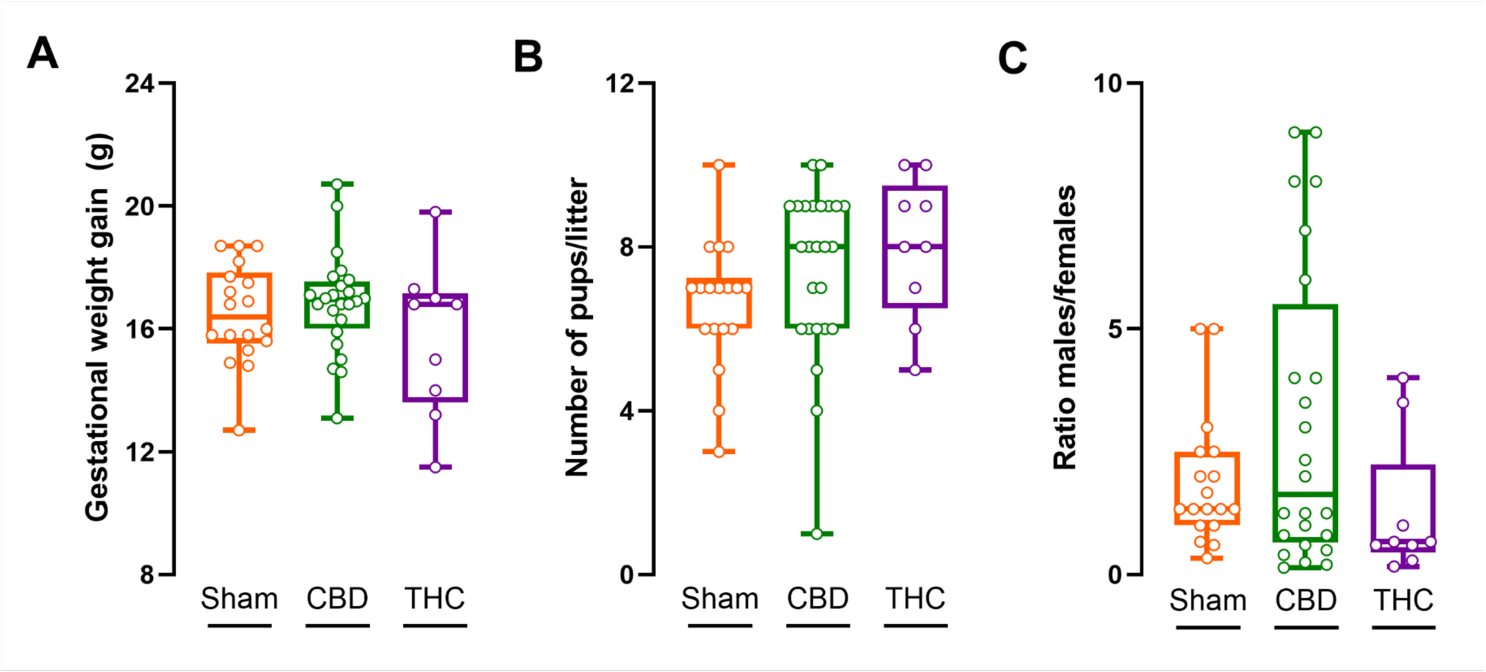

Figure 1

Prenatal CBD and THC exposure does not alter maternal weight gain or reproductive outcomes. (A) Maternal weight gain throughout gestation. (B) Litter size at birth. (C) Offspring sex ratio. Prenatal exposure to CBD or THC had no significant effect on maternal physiological or reproductive parameters. Data are presented as box-and-whisker plots showing median, minimum, and maximum values; each point represents a single litter (Sham 18; CBD 25; THC 8). Statistical comparisons were performed using the **Kruskal-Wallis test: $H_{(weight\ gain,\ 2)}$= 1.601, p-value = 0.449; $H_{(\#pups,\ 2)}$= 5.02, p-value = 0.081; $H_{(ratioMF,\ 2)}$= 3.548; p-value = 0.169**. Only results with $p < 0.05$ were considered statistically significant and reported.

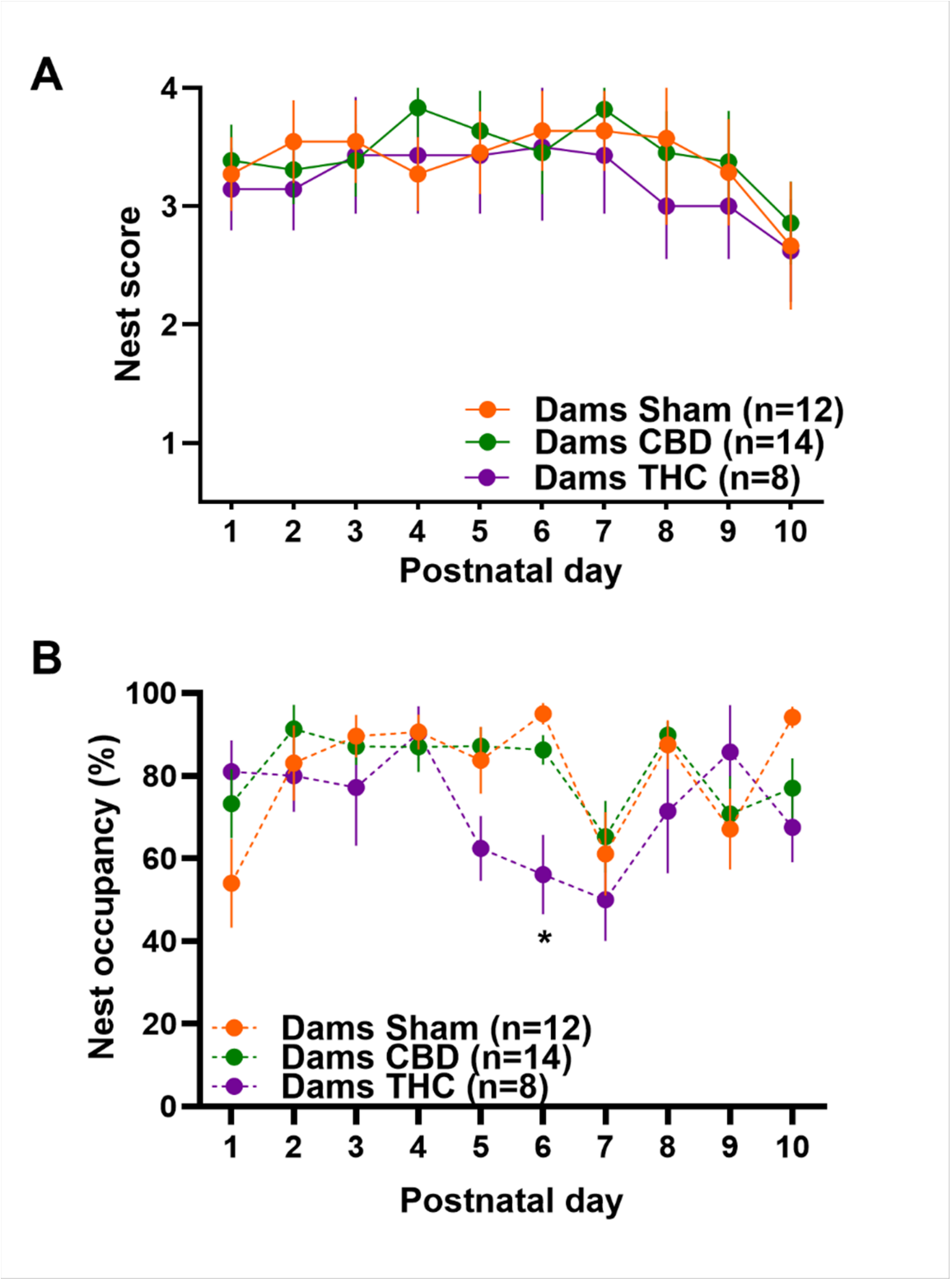

Figure 2

Prenatal THC, but not CBD, exposure transiently alters maternal nest occupancy. (A) Daily assessments of nest quality indicate that prenatal CBD or THC exposure does not disrupt nest-building behavior, which remains comparable to vehicle-treated controls. (B) Daily 20-min home-cage observations reveal a consistent preference for nest occupancy during the early postnatal period; however, THC-exposed dams exhibit a transient reduction in nest's occupancy at PND 6. Data are expressed as mean ± standard error

of the mean (SEM). Statistical analysis was performed via mixed-effects models with the Geisser-Greenhouse correction, followed by Tukey's post hoc multiple comparison test: A ($F_{(time, 5.26, 127.9)}$ = 6.417, **p-value<0.0001; $F_{(treatment, 2, 29)}$ = 2.370, p-value = 0.113, $F_{(interaction\ 10.52, 127.9)}$ = 1.066, p-value = 0.393**), B ($F_{(PND\ 5.08, 152.4)}$ = 3.743, **p-value = 0.003; $F_{(treatment\ 2, 31)}$ = 2.107, p-value = 0.139; $F_{(interaction\ 10.16, 152.4)}$ = 1.761, p-value = 0.071**). Only results with $p < 0.05$ were considered statistically significant and reported.

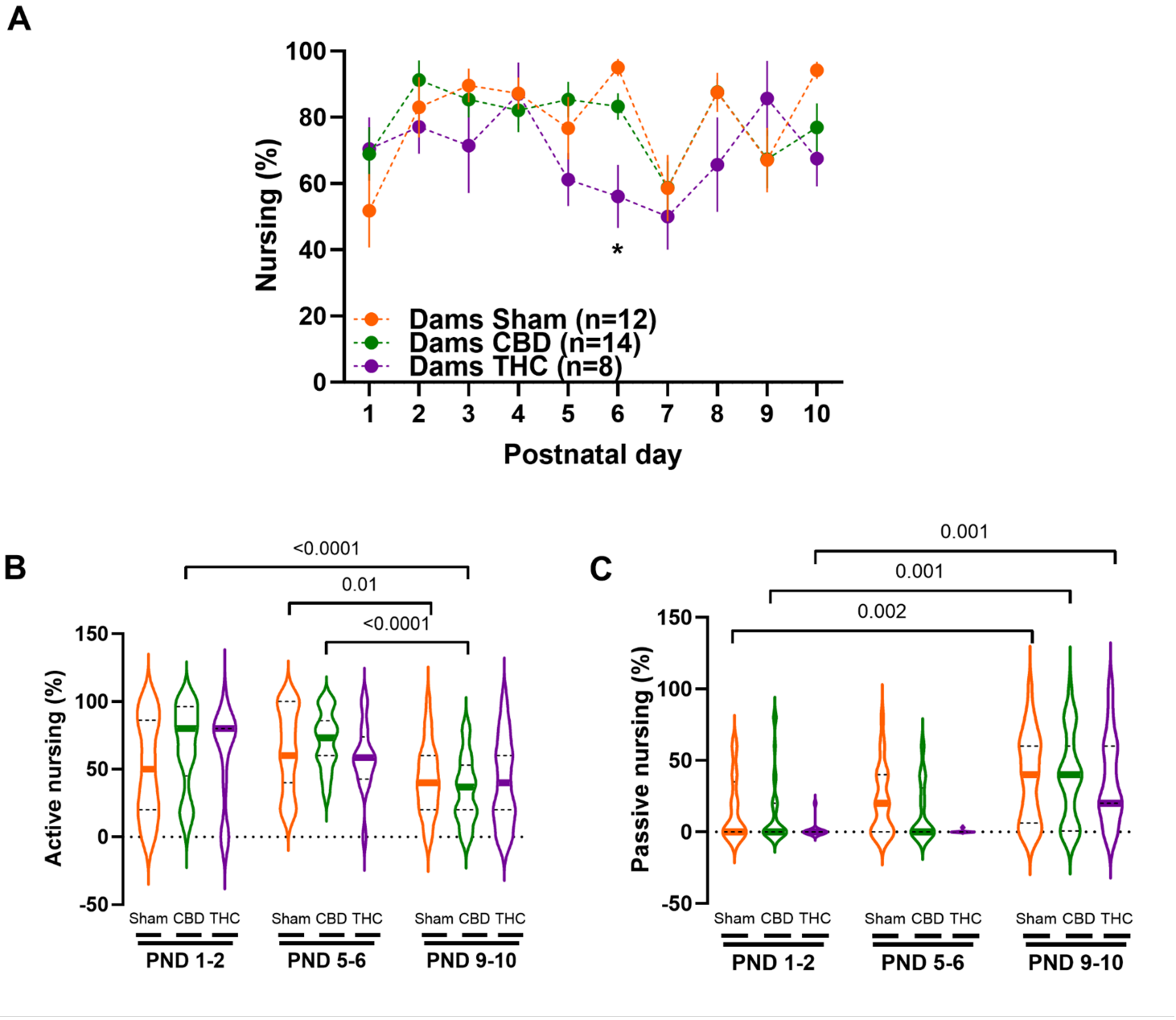

Figure 3

Prenatal THC exposure transiently alters nursing duration, while nursing style shifts from active to passive over postnatal development. (A) Time-course analysis of daily nursing duration across PND 1–10. THC-exposed dams exhibit a transient reduction in nursing duration at PND 6, concurrent with the

nest occupancy deficit (see Figure 2B); otherwise, nursing duration remains consistent across groups. (B–C) Nursing style classification, showing the predominance of (B) active nursing in the early postpartum period and the transition to (C) passive nursing by the end of the follow-up (PND 9–10). (A) Data are presented as mean ± SEM in XY plots or as (B–C) violin plots indicating minimum, maximum, and median values. Statistical analysis was performed via **mixed-effects models** with the Geisser-Greenhouse correction, followed by Tukey's post hoc multiple comparison test: A ($F_{(PND\ 5.121,\ 153.6)}$ = 4.151, **p-value = 0.001; $F_{(treatment\ 2,\ 31)}$ = 2.293, p-value = 0.118; $F_{(interaction\ 18,\ 270)}$ = 1.613, p-value = 0.056**). B ($F_{(PND\ 2,\ 127)}$ = 16.08, **p-value <0.0001; $F_{(treatment\ 2,\ 65)}$ = 0.4829, p-value = 0.619; $F_{(interaction\ 4,\ 127)}$ = 1.496, p-value = 0.207**). C ($F_{(PND\ 1.757,\ 111.6)}$ = 35.25, **p-value <0.0001; $F_{(treatment\ 2,\ 65)}$ = 2.654, p-value = 0.078; $F_{(interaction\ 4,\ 127)}$ = 1.143, p-value = 0.339**). Only results with $p < 0.05$ were considered statistically significant and reported.

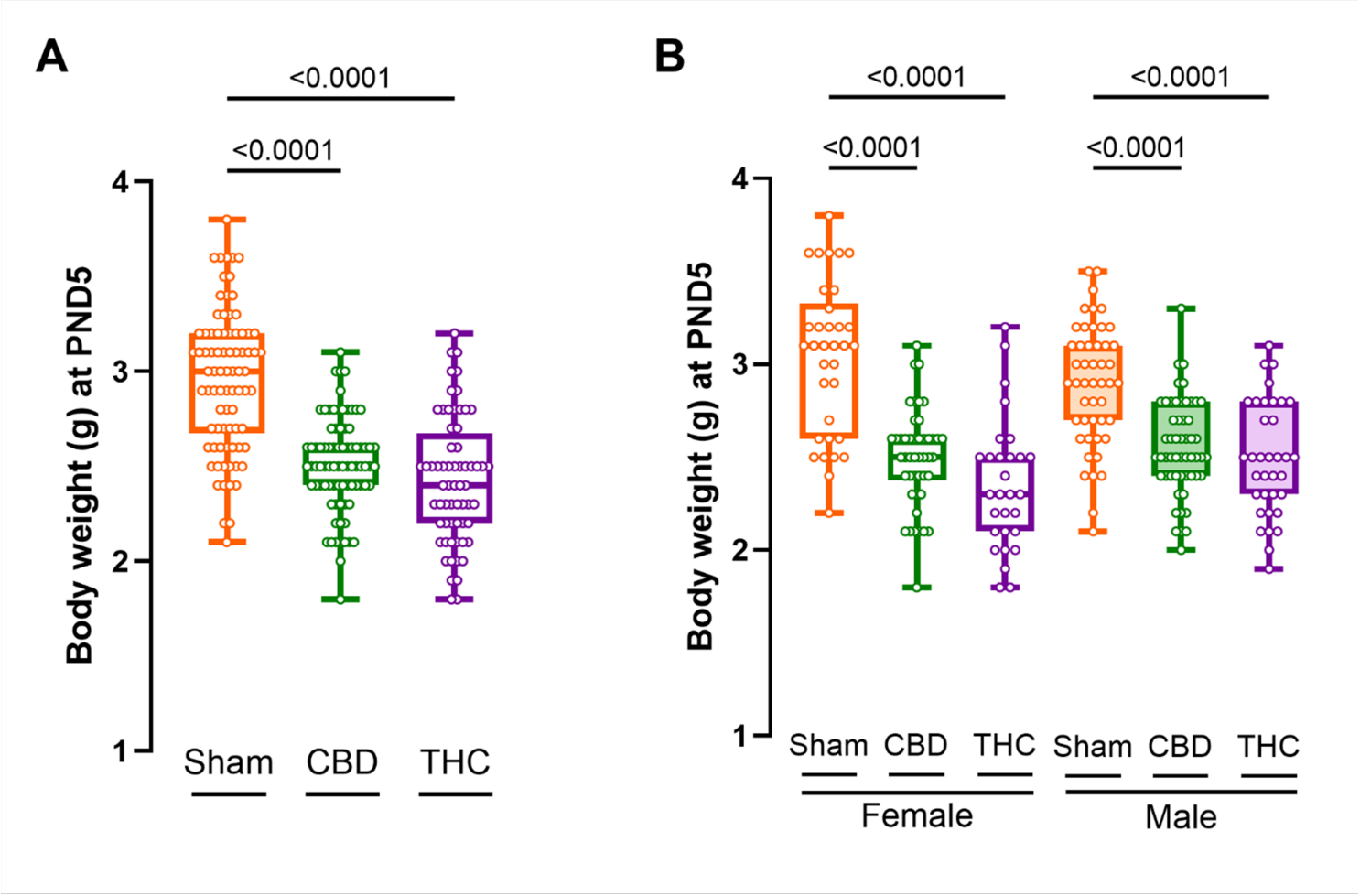

Figure 4

Prenatal CBD and THC exposure causes a sex-independent reduction in body weight at PND 5. (A) Comparison of pup body weight at PND 5, showing significantly reduced weight in both CBD- and THC-exposed groups relative to Sham controls. (B) Analysis of body weight by sex reveals that the growth restriction is consistent across males and females, confirming a sex-independent phenotype. Data are presented as mean ± SEM. Group sizes: Sham (females , males), CBD (females , males), and THC

(females , males). Statistical analysis: **(A)** one-way ANOVA ($F_{(treatment\ 2,\ 226)}$ = 60.91, p-value <0.0001) and **(B)** two-way ANOVA followed by Tukey's post hoc multiple comparison test (**$F_{(interaction\ 2,\ 240)}$ = 3.801, p-value = 0.0237**), $F_{(sex\ 1,\ 240)}$ = 0.294, **p-value = 0.588; $F_{(treatment\ 2,\ 240)}$ = 65.98, p-value <0.0001**). Only results with $p < 0.05$ were considered statistically significant and reported.

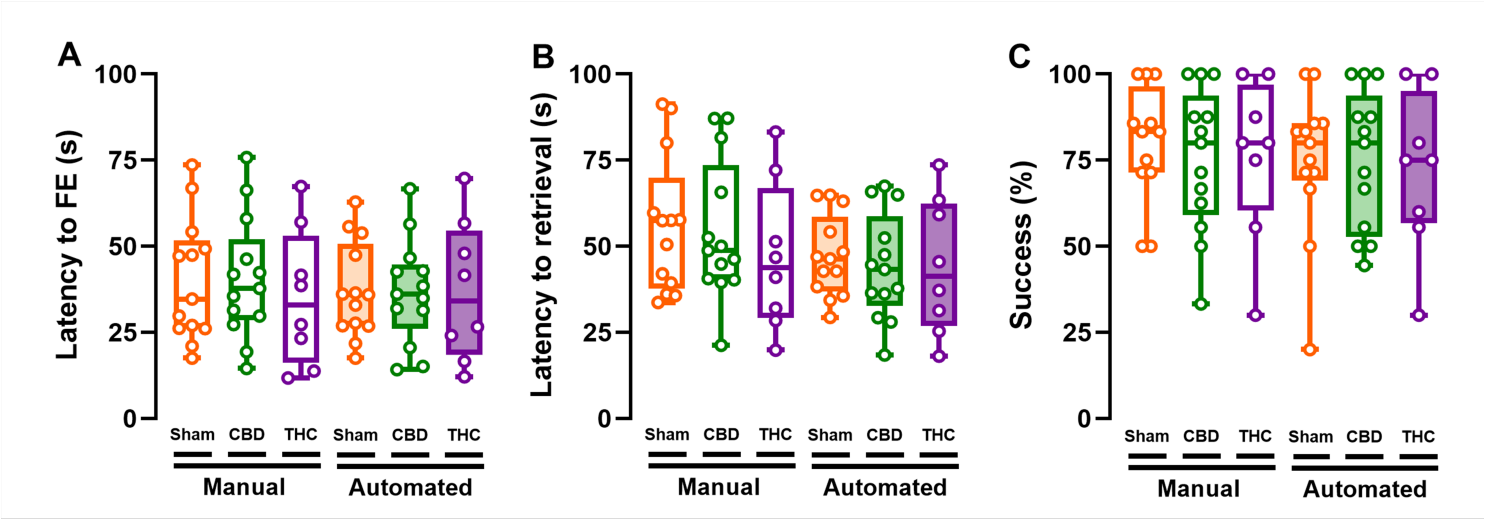

Figure 5

Prenatal CBD or THC exposure does not alter mean per-litter maternal behavior. (A) Latency to pup first encounter (FE), (B) retrieval response time, and (C) overall retrieval performance were unaffected by prenatal CBD or THC exposure when analyzed as mean per-litter values. Manual (left) and automated (right) scoring methods yielded consistent results. Data are presented as box-and-whisker plots showing median, minimum, and maximum values; each point represents the mean dam performance across all pups within a single litter. Group sizes: Sham (13), CBD (13), and THC (8). Statistical analysis was performed using **two-way ANOVA** followed by Tukey's post hoc multiple comparison test: A ($F_{(interaction\ 2,\ 62)}$ = 0.1416, **p-value = 0.8682**), $F_{(type\ of\ detection\ 1,\ 62)}$ = 0.1419, **p-value = 0.7077; $F_{(treatment\ 2,\ 62)}$ =0.1392, p-value = 0.8704**); B ($F_{(interaction\ 2,\ 62)}$ = 0.3031, **p-value = 0.7396**), $F_{(type\ of\ detection\ 1,\ 62)}$ = 0.2.867, **p-value = 0.0954; $F_{(treatment\ 2,\ 62)}$ =0.4735, p-value = 0.4735**); C ($F_{(interaction\ 2,\ 62)}$ = 0.0693, **p-value = 0.9331**), $F_{(type\ of\ detection\ 1,\ 62)}$ = 0.029, **p-value = 0.8662; $F_{(treatment\ 2,\ 62)}$ =0.0179, p-value = 0.9824**). Significance was set at $p < 0.05$.

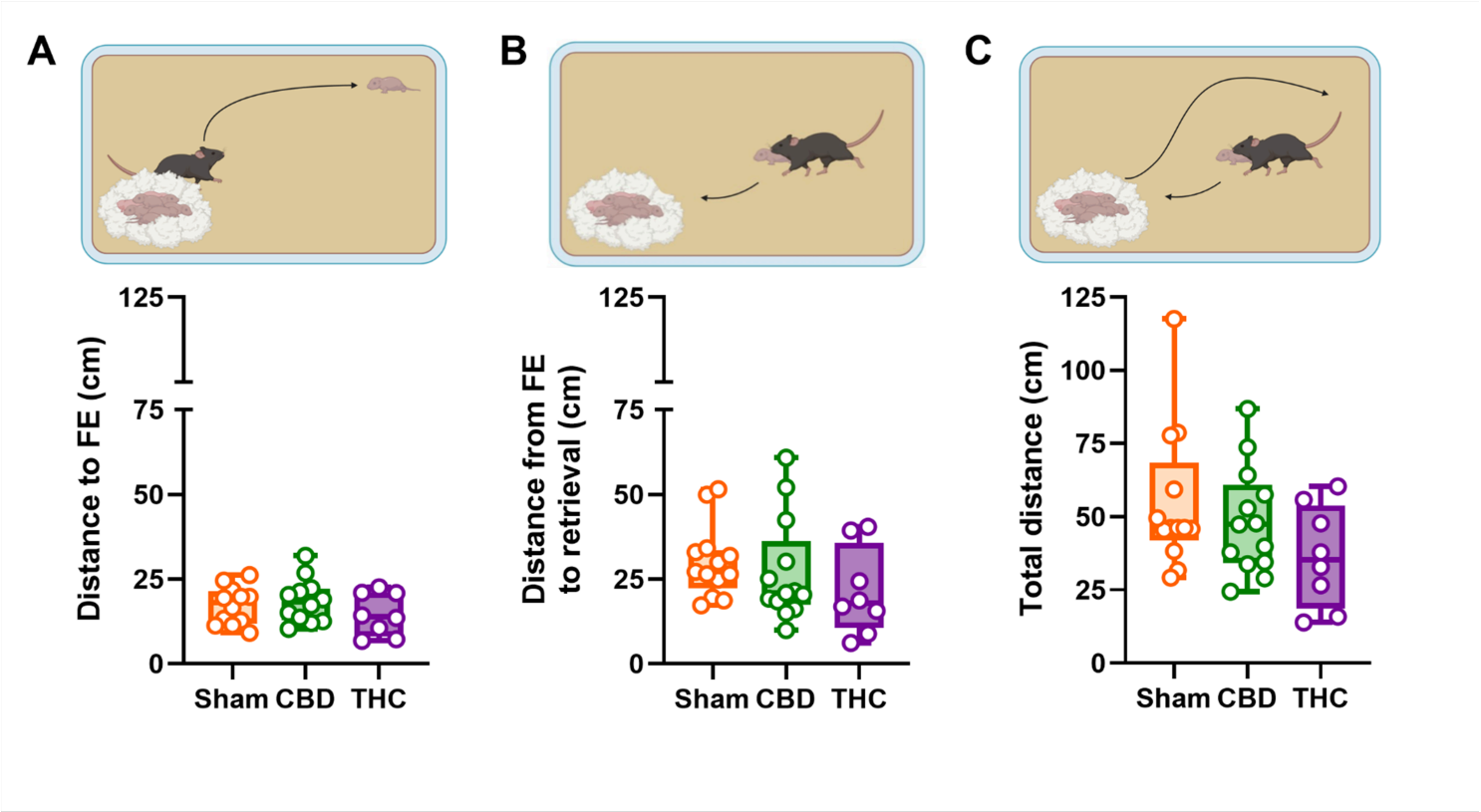

Figure 6

Prenatal CBD or THC exposure does not alter locomotor performance during the pup retrieval task (PRT). (A–C) Quantification of movement patterns across groups. There were no significant differences in the (A) distance traveled to locate pups, (B) distance covered during pup retrieval, or (C) total distance traveled during the complete retrieval sequence. Data are presented as box-and-whisker plots showing median, minimum, and maximum values; each data point represents the mean performance per dam across all trials. Group sizes: Sham (n = 13), CBD (n = 13), THC (n = 8). Statistical analysis was performed using the **Kruskal-Wallis test: $H_{(dFE,\ 2)}$= 1.253, p-value = 0.5346; $H_{(dFE\text{-}R,\ 2)}$= 3.898, p-value = 0.1424; $H_{(total\ distance,\ 2)}$= 2.586, p-value = 0.2745,** with significance defined as $p < 0.05$.

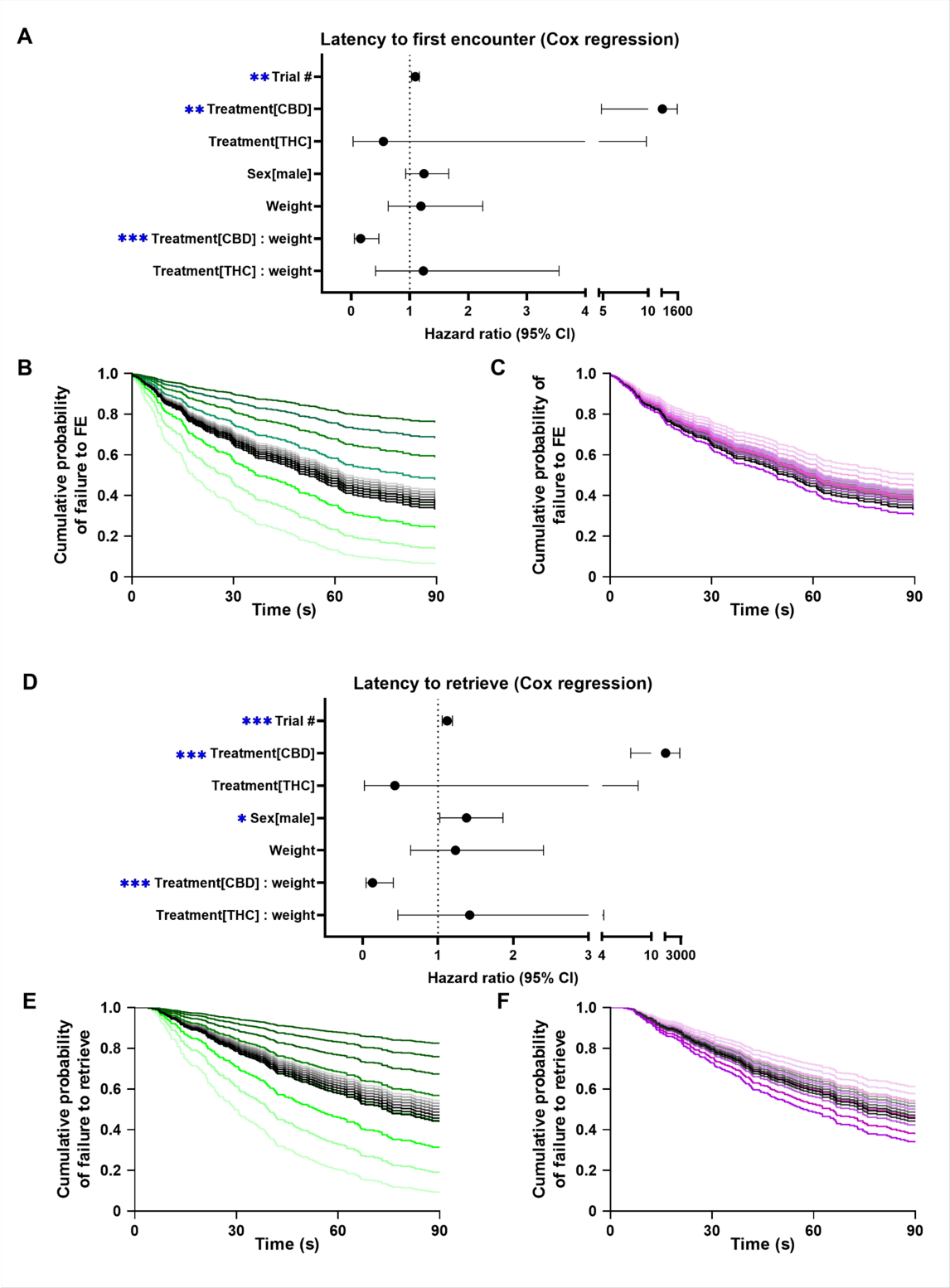

Figure 7

Maternal responses to CBD-exposed pups are modulated by pup body weight. (A, D) Forest plots displaying hazard ratios (HR) and 95% confidence intervals (CI) from multivariate Cox proportional hazards (Cox-PH) models; an HR>1 indicates an increased hazard (faster rate) of event completion. (B–C, E–F) Predicted cumulative probability of task non-completion over time, modeled across a weight gradient (1.8–3.2 g in 0.2 g increments; shading intensity increases with weight). Color key: Black =

Sham, Green = CBD, Purple = THC. (A–C) Pup encounter: (B) CBD treatment increases the likelihood of encounter in a weight-dependent manner, where lighter pups are encountered more rapidly; (C) Sham and THC groups exhibit weight-independent encounter latency. Pup order in the test is a significant covariate, indicating a learning effect. (D–F) Pup retrieval: (E) CBD treatment enhances retrieval likelihood, moderated by body weight; (F) No weight-dependent effect is observed in the THC group, despite comparable growth restriction (see Figure 4). Analysis includes 82 control pups (13 litters), 100 CBD-exposed pups (13 litters), and 64 THC-exposed pups (8 litters).

Supplementary Files

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- SupFG2.png
- SupFG3.tif
- SupFG4NEST.tif
- SupFG5.tif
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