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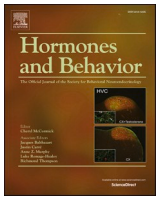

Postnatal rearing environment alters pup cues for caregiver-offspring interactions

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ABSTRACT

Maternal behavior experienced in early life provides essential scaffolding to infant psychobiology with life-long effects on neurobiological and behavioral outcomes. However, infants are not passive recipients of caregiving. Evidence in rodents suggests that pups actively contribute to dam-pup interactions by soliciting maternal care with auditory, tactile, and hormonal cues. The limited bedding and nesting material (LBN) rearing manipulation induces changes in maternal care that have been attributed to maternal stress caused by the low-resource environment. The goal of the current study was to determine whether LBN also alters pup cues for maternal behavior, with implications for the mechanism of LBN-induced effects. Rat dams and pups were randomly assigned to LBN or Control rearing conditions on postnatal day (P) 0–6 and pups were fostered to the same or different condition on P6–13. LBN increased pup-directed maternal behaviors measured through 24 h monitoring using machine learning based automated analysis. LBN altered several pup cues known to affect maternal behavior including reducing pup core body temperature, reducing body weight, and altering pup vocalizations on P6 and P12. P6–13 LBN-exposed pups had elevated serum testosterone, which positively correlated with maternal licking and grooming. LBN reduced pup movement between nest attendance onset and the start of nursing, which was negatively related to dam nursing latency and contributed to longer nursing latency in LBN dams. P0–6 pup exposure to LBN also led to longer nest attendance bouts and shorter licking and grooming bouts on P7 and P9, suggesting lasting effects of LBN on pups. These data demonstrate that LBN changes pup behavioral and hormonal signals consistent with eliciting more maternal care, contributing to augmented pup-directed behaviors. This bidirectional interplay may be a critical mechanism involved in the lasting effects of early life environments.

1. Introduction

The perinatal period is a time of elevated plasticity within the developing brain. In mammals, this period is characterized by parental interactions with offspring which are predictive of physical health, cognition, social behavior, and emotional regulation across the lifespan (Kundakovic and Champagne, 2015). Though variation in maternal care and it's "programming effects" have been widely considered the mechanism of these long-term outcomes (Curley and Champagne, 2015), maternal-offspring interactions are not unidirectional. Infants are active participants in promoting mother-infant attachment with infant behavior facilitating maternal attention and progressive development of dyadic interactions. Decades of human studies examining coordination of caregiver-offspring interactions have demonstrated that bidirectional physiological and behavioral responsiveness is associated with attachment, social, emotional, and cognitive outcomes for offspring, underscoring the importance of understanding dyadic interaction in early life (Beeghly and Tronick, 2011; Feldman, 2007; Jaffe et al., 2001).

Bidirectional caregiver-offspring interactions are not unique to humans. In laboratory rats, dams alter pup-directed maternal care across the pre-weaning period driven by the size and age of pups, rather than time since parturition, to provide developmentally-appropriate maternal care (Moore, 2007; Rosenblatt, 2003; Stern and Mackinnon, 1978). Temperature of pups also affects maternal care such that dams spend more time attending to cool pups compared to warm pups (Leon et al., 1978). Perhaps the most well-studied pup cue is pup ultrasonic vocalizations (USVs), which vary with pup age, are affected by environmental exposures, and attract parental attention (Brudzynski et al., 1999; Moore, 2007; Rosenblatt, 2003). Similarly, tactile stimulation provided by pups to the dam's ventrum through nipple attachment and

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suckling induces the dam to assume an active nursing posture and facilitates milk letdown (Stern, 1996). Latency to initiate the nursing posture is dependent on summative tactile input from pups, which is influenced by pup body size and the number of pups in the litter (Stern and Johnson, 1989, 1990).

The reciprocal nature of the mother-infant relationship in rodents is also highlighted by the exchange of resources during pup licking by the dam. Pups are unable to urinate independently in early life and tactile anogenital stimulation provided by the dam allows for pup urination while dams benefit by ingesting the dilute pup urine (Alberts and Gubernick, 1983). Maternal pup-directed anogenital licking is affected by appetite for water and electrolytes found in the urine, which may be subject to environmental influences (Gubernick and Alberts, 1983). Moreover, the amount of licking and grooming a particular pup receives is affected by behavioral and physiological signals provided by the pup. For example, shorter latency of pup leg-extension response during anogenital licking and grooming and longer latency to urinate contribute to male pups receiving more licking and grooming than female pups (Clark et al., 1989; Moore and Chadwick-Dias, 1986). Dams are also drawn to chemical signals dependent on pup testosterone levels that make urine from male pups more attractive (Moore, 1982; Moore and Morelli, 1979). Licking and grooming duration is affected by pup body position (supine/prone, reverse orientation) and inhibition of pup righting-response during licking and grooming, which change with pup age (Moore and Chadwick-Dias, 1986). While it is clear that pup cues guide maternal-pup interactions across several modalities, these signals are generally underexplored in studies examining the effects of developmental exposures on maternal care and offspring development.

Environmental exposures that alter dam-pup interactions in early life have lasting physiological effects on pup development that can endure into adulthood and may lead to intergenerational effects (Champagne and Meaney, 2001). Caregiver-offspring interactions play a foundational role as mediators and moderators of early life experience. Factors beyond the infant's proximal environment can influence the infant indirectly through parental care and parents can also buffer offspring from negative effects of adverse early experiences through changes in caregiving behavior. One context in which the effects of the environment on dam-pup interactions have been studied extensively in rodents is the limited bedding and nesting (LBN) material environmental manipulation of early adversity, in which the dam and litter is supplied with little bedding material in the home cage (Gilles et al., 1996; Roth and Sullivan, 2005). Various versions of LBN have been implemented and effects on maternal care vary with the specific methodology used (Walker et al., 2017). Overall, these studies have identified immediate and enduring effects of LBN on offspring including disrupted social behavior, changes in anxiety and depression-like behavior, memory deficits, altered reward neurocircuitry, and dysregulated hypothalamic-pituitary-adrenal physiology (Walker et al., 2017). These neurodevelopmental effects are largely assumed to be the consequence of LBN-induced increases in maternal stress caused by the low resource environment, which in turn alter pup-directed maternal care.

In addition to the direct effects of the LBN environment on dams, this rearing paradigm alters the pup's proximal environment in the home cage with potential to impact pup cues for maternal behavior. Therefore, disrupted maternal behavior observed with LBN may result both from the direct effect of LBN on dams and through alterations to pup behavioral and physiological cues for maternal behavior, which in turn contribute to altered maternal care. Previous work has shown that during LBN, pups gain less weight, huddle less cohesively in the home cage, and have differential brown fat activation compared to Control pups consistent with a cooler nest microclimate, suggesting that LBN-induced changes in pup behavior and physiology may contribute to LBN-induced disrupted maternal care (Lapp et al., 2020b). The goals of the present study were to 1) examine acute effects of LBN on pup cues for maternal behavior including pup core body temperature, pup vocalizations, and pup testosterone levels during LBN exposure; 2) conduct a detailed analysis of bidirectional home cage dam and pup behavior during LBN by implementing an automated behavioral pipeline (Lapp et al., 2023); and 3) investigate whether pup exposure to LBN leads to lasting changes that affect maternal behavior after the manipulation has ended. To test for lasting LBN-induced changes, we implemented a cross-over experimental rearing design to allow for assessment of the impact of prior LBN exposure on dam-offspring interactions.

2. Methods

2.1. Animal husbandry and breeding

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at Austin and were performed in accordance with IACUC guidelines and regulations. Animals were housed in polycarbonate cages (48 cm × 26.5 cm × 20 cm) with standard wire tops and were kept on a reverse 12:12 h light cycle (lights off at 10 am EST). All dams were provided with Aspen shavings (Nepco) for bedding material, which can be manipulated by dams to construct nests. No other bedding material was provided. All animals were fed standard chow (Lab diet 5LL2) and water ad libitum. Sixty adult Long-Evans female rats and 30 adult Long-Evans male rats were purchased from Charles River Labs and acclimated to the vivarium for at least two weeks before breeding. During breeding, females were screened daily for receptive behavior and housed with a breeder male overnight on the day lordosis was observed. All dams were socially housed throughout pregnancy until they were separated into individual cages a few days before giving birth.

2.2. Experiment time line

Day of birth was considered postnatal day (P) 0. On P0, pups were culled to five males and five females per litter (sex determined using anogenital distance) and litters that did not meet this minimum criterion were excluded from the study. Dams and litters were randomly assigned to Control (225 g of bedding) or LBN (50 g of bedding) conditions immediately following litter culling (adapted from Moriceau et al., 2009; Fig. 1A). A subset of dams ($n = 7$) in the LBN condition used all available food as supplemental bedding material. Food was removed from the bottom of the cage when food hoarding was observed. Biotherm tag implantation took place on P3 or P6 (Fig. 1B). On P6, pups underwent UVS recording testing (5:00 am to 9:00 am). All litters were fostered to a new dam in a clean cage in the same or opposite condition at the end of P6, where they remained undisturbed until P12. Dams remained in the same condition for the duration of the experiment. On P12, pup USV recordings were collected (5:00 am to 9:00 am). Serum was collected from pups on P13 (4:00 pm to 9:00 pm).

2.3. Video recording and home cage behavior analysis

Home cage behavior was recorded with Raspberry Pi 3B+ mini-computers running Debian bullseye with the Raspberry Pi Desktop and equipped with Raspberry Pi Module 1 NoIR cameras as previously described (Lapp et al., 2023). 24-hour recordings at 2 frames per second in greyscale were taken starting 8 h after lights-off on P1, P3, P7, and P9. In the event that the dam moved the location of the nest to the opposite end of the cage, the camera side was also switched at the first opportunity.

Prior to analysis of maternal behavior, videos were screened to check that the camera was placed at the nest end of the cage and the dam did not move the nest to the opposite cage end during the recording. Videos were automatically scored using the AMBER pipeline (Goodwin et al., 2024; Lapp et al., 2023; Lauer et al., 2022; Mathis et al., 2018). AMBER behavior annotations are at the level of each frame and behavior annotation data was postprocessed (see **Supplemental methods**). Next, total duration, bout duration, and bout intervals were calculated for

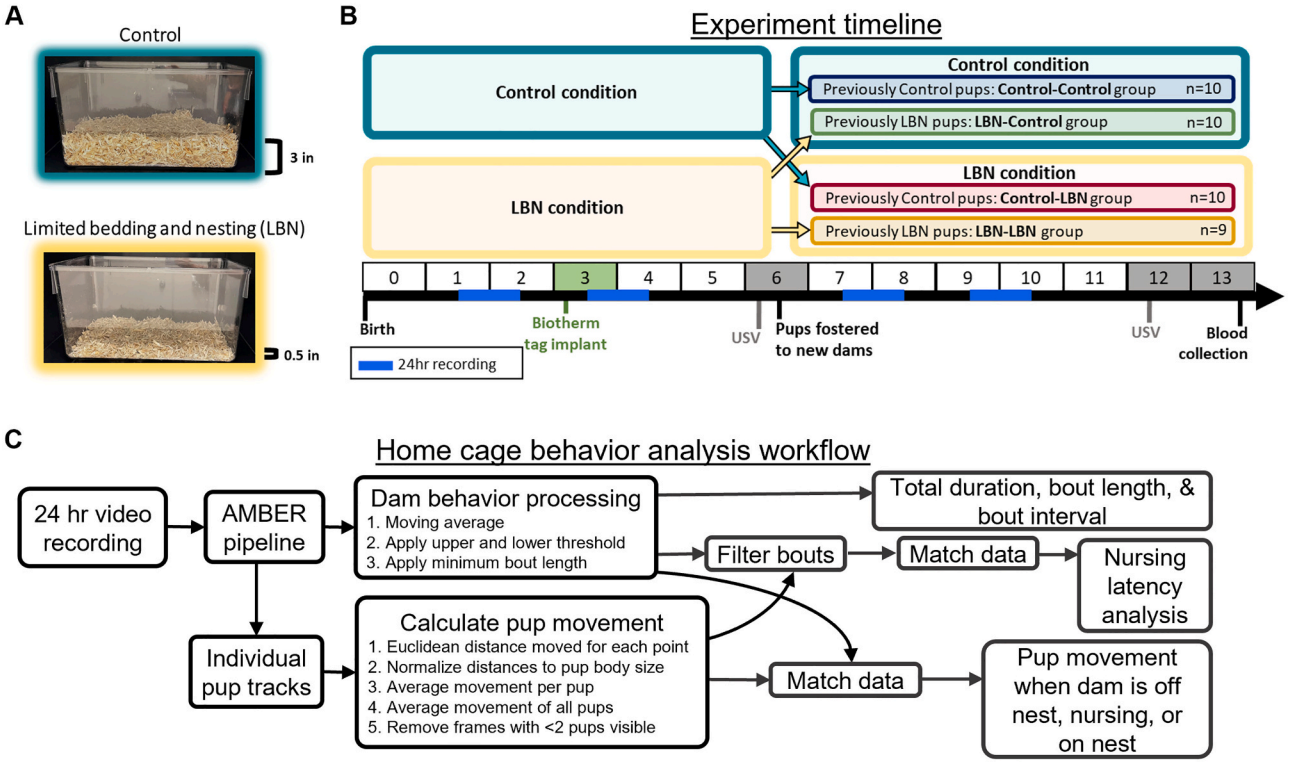

Fig. 1. Experimental design. A. Dams and litters in the Control condition received 225 g and dams and litters in the LBN condition received 50 g of aspen shaving bedding material. B. Experiment timeline. Litters were culled and dams and litters were assigned to Control or LBN conditions on day of birth. Pups were implanted with BioTherm tags on P3 or P6. Pups were fostered to a dam in the same or opposite condition on P6. Pup ultrasonic vocalization recordings from individual pups were taken on P6 and P12. 24 h home cage video recordings were taken starting on P1, 3, 7, and 9. Trunk blood was collected from pups on P13. C. Home cage behavior analysis workflow. The AMBER pipeline was used to analyze home cage recordings for nest attendance, nursing, and licking and grooming maternal behavior. Dam behavior was post-processed and total duration, bout length, and bout intervals were calculated. Frame-frame pup movement while the dam was off nest, nursing, and on nest (but not nursing) was analyzed by assembling individual pup tracks in DeepLabCut and calculating average normalized movement for all tracked pups in R. Pup movement data sets were matched for the number of pup point tracked and number of individual pups tracked. Nest attendance bouts were filtered to those lasting at least 60 s with no nursing in the preceding 30 s to assess effects on time to begin nursing after nest attendance onset (nursing latency).

each behavior for each video (Fig. 1C).

Pup movement was calculated by assigning pup detections identified by the AMBER pipeline to individual pups in DeepLabCut using the create_tracklets and stitch_tracklets functions (Fig. 1C). Euclidean distance of each individual body part detected across frames was calculated. Distance moved for each body part in pixels was normalized by dividing by the median Euclidean distance from the pup nose point to the pup eye point across all frames for that video. This is an imperfect normalization step to account for pup body size and location of the pups relative to the position of the camera within each video. Distances moved between frames that were >1.5 times the normalization distance were assumed to result from poor tracking or identity swaps and were removed; thus, the maximum distance any pup point could move in 0.5 s was 1.5 the median distance from pup nose to eyes. Next, the normalized average distance moved of all visible points for each individual pup were averaged for each frame to produce an average movement measure per pup. Pup movement for the entire litter was then calculated by averaging the distance moved of all visible pups in the litter for each frame.

Pup tactile stimulation of the dam's ventrum is known to promote active nursing posture, where higher summative levels of stimulation by the litter reduce the length of time the dam takes to begin quiescent nursing after the onset of interaction with pups, here referred to as nursing latency. We first examined nursing latency to see if rearing condition affected dam latency to begin nursing. Next, we explored the relationships between pup movement during this period, exposure to LBN, postnatal day, and nursing latency to determine whether: a) pup movement predicted nursing latency and b) whether LBN exposure affects the relationship between pup movement and nursing latency. Only nursing latency bouts where the dams had not been on the nest for at least 30 s preceding the bout and the dam was on the nest continually for at least 60 s were included in the analysis.

2.4. Pup body temperature

One male and one female pup from each litter was implanted with a temperature passive integrated transponder Biotherm tag (BioTherm13, Biomark) on P3 (20 litters) or P6 (19 litters) on their flank using a 12-gage needle. Needle insertion sites were closed using tissue adhesive. After the adhesive dried, pups were placed back in the nest in their home cage. Pilot studies showed that pups with implanted Biotherm tags were accepted by the dam and were not treated differently than pups without implants after return to the home cage (unpublished observations). Pup core body temperatures were measured using a handheld reader (HPR Lite Handheld Biotherm Tag Reader, Biomark) held outside of the home cage to read the identifying tag number and temperature of each tagged pup. Temperature measurements were taken a minimum of eight times per day without disturbing dams or pups with each reading at least 1 h apart. Pups implanted with Biotherm tags were not used for USV recordings or blood collection.

2.5. USV recordings

One male and female pup from each litter was subject to USV testing on P6 and P12. Pups were taken from their home cage and placed on a heating pad for up to 10 min prior to testing. Pups were then individually placed in a glass arena (20 cm × 20 cm) in a sound-attenuated

chamber with acoustic foam and an ultrasonic microphone placed 10 cm above the arena (#40011, Avisoft). Vocalizations were recorded for 10 min. Pups were weighed then immediately returned to their home cage.

2.6. Ultrasonic vocalization analysis

Pup calls were identified using DeepSqueak and the Rat detector YOLO R1 network to detect calls (Coffey et al., 2019). Detected calls were manually reviewed and calls with multiple fundamental frequencies (biphonation or harmonic calls) were manually labeled. Call features were exported as csv files and call data was analyzed in R. The total number of calls, average call duration, sum duration of all calls, and time of first biphonation call were calculated. In addition, calls were classified by type into flat calls (<8 kHz delta frequency), short calls (<80 ms), very short calls (<25 ms), audible calls (<20 kHz principal frequency), 60 kHz calls (principal frequency > 55 kHz), and complex calls (longer calls with >8 kHz delta frequency) according to extracted call features. There were very few audible calls and 60 kHz calls during P12 recordings, so they were excluded from P12 analysis. The principal frequency of calls excluding 60 kHz calls, audible calls, and biphonation calls, was also assessed.

2.7. Pup serum testosterone

Pup testosterone levels regulate the amount of maternal licking and grooming they receive, so we explored whether serum testosterone was affected by LBN exposure and related to maternal licking and grooming (Moore, 1982; Moore and Morelli, 1979). Whole blood was collected from pups at the conclusion of the experiment on P13. Trunk blood from one male and one female was collected after decapitation. Blood was spun down at 1000 x*g* for 10 min at least 30 min after collection to separate serum. Serum was aliquoted and stored at —80C until analysis. Serum testosterone was measured with ELISA (ADI-901-065, Enzo Life sciences) according to the manufacturer's instructions.

2.8. Statistics

All data was analyzed using linear mixed models in R with the lmerTest package. Data that did not meet the assumption of normality was scaled or log transformed as noted. Two separate analyses were run for temperature and home cage behavior data. The first analysis included data collected before pups were fostered to new dams on P6 with condition included as a factor in the model. The second analysis was run on data collected after P6 and included pup previous condition (P0–6 condition) and the condition the pups and dams were in at the time of measurement. For clarity, "concurrent condition" refers to the condition dams and pups were in at time of measurement across the experiment. Covariates included in models were temperature in the housing room at time of measurement for pup body temperature, postnatal day for home cage maternal behavior, use of food as supplemental bedding material for maternal behavior, and the number of pup points used to calculate movement for pup movement. For USV analysis, pup body weight, time separated from the dam prior to testing, and testing order were included in the model. Dam/litter ID and/or pup ID were included as random factors in models as appropriate. The best model (e.g. including or excluding covariates, including or excluding interaction terms) for each analysis was selected by identifying the model with the lowest Akaike Information Criteria (AIC) values. Results are reported as significant when $p < .05$ and reported as trends when $p < .1$.

A single pup movement value was calculated for each frame (Euclidean distance of a given body point from one frame to the next frame) by first calculating the mean movement of all tacked points for each pup tracked then calculating the average movement of all pups tracked in that frame. The number of pup points tracked by the AMBER pipeline was larger when the dam was off nest and increased with pup age. Pups were not equally tracked between conditions as bedding is more likely to occlude pups in the Control condition compared to the LBN condition. There were more overall frames with pup tracking in the LBN group compared to the Control group and the mean number of points tracked was larger in the LBN condition, particularly at older ages (**Supplemental Fig. 2**). Similar patterns exist for the number of individual pups tracked in each frame (**Supplemental Fig. 1**).

To minimize the effect of unequal tracking of pups between conditions, matched data sets were created that balanced both the number of individual pups tracked and the total number of pup points tracked for each frame with the MatchIt package in R. The MatchIT package is designed to preprocess data to enable analysis of an exposure on an outcome while controlling for confounding variables to ensure the resulting exposure effect estimate is not influenced by the confounding variables to allow for interpretation as a causal effect using parametric models (Ho et al., 2011). Creating a matched data set aims to produce distributions in covariates, in this case the number of pup points tracked and number of individuals pups tracked in each frame, that are equal between groups as they would be in a randomized experiment (Ho et al., 2007). To accomplish this, separate data sets were created for pup movement for each postnatal day and dam status (on nest, off nest, or nursing) using the "quick" (recommended for large data sets) and "glm" distance options in MatchIt. Quality of matched data was determined using standard mean difference, empirical cumulative distribution function (eCDF), and variance ratio statistics. In all cases, the matched data sets for LBN and Control groups were well balanced for both the number of individual pup points tracked and number of individual pups tracked as indicated by standard mean difference and eCDF values close to zero and variance ratio values close to 1 (see **Supplemental Table 1**). Pup movement analyses were performed for matched data sets and for all collected data using the number of pup points tracked as a covariate. Pup movement analysis results did not change if the number of individual pups tracked was used as a covariate instead of the total number of pup points.

Similar methods were used to create balanced datasets for pup movement during nursing latency. Instead of individual frames, nursing latency data sets for each postnatal day were created by balancing the mean number of pups and mean number of pup points tracked during the nursing latency bout. Using bout, rather than individual frames, as the matching unit permitted use of the nearest neighbor matching (method = "nearest") for data sets created on P1, 3, and 7. Nearest neighbor matching did not produce a well-balanced dataset for P9 data, so coarsened exact matching was used (method = "cem"), which first divides the variables into bins, preforms exact matching, drops unmatched observations, then reweights the remaining observations (**Supplemental Table 1**).

Three nursing latency mixed models were generated using matched pup movement data: one model encompassing all home cage data, one model with only P3 data, and one model with only P9 data and pup previous condition as a fixed factor. Pearson's correlations were run as post-hoc analyses to examine the variation in the relationship between pup movement and nursing latency within each group on each postnatal day.

To complement the mixed model analysis of nursing latency and pup movement, these data were also analyzed using path analysis (structural equation modeling) with the lavaan and piecewiseSEM packages (Rosseel, 2012; Lefcheck, 2016). This analysis approach allows for assessment of all relationships among variables at once and allows variables to be both predictor and response variables. The piecewiseSEM function *psem* also allows for inclusion of random factors (Dam/litter ID). Adequate global model fit was evaluated using the $\chi 2$ statistic ($p > .05$) or Fisher's *C* statistic if submodels failed to converge and the $\chi 2$ statistic was not reported. AIC was used to compare models. The path models included the same factors used for the mixed model approach with a few exceptions. First, condition variables were dummy coded as "0" for Control and "1" for LBN since piecewiseSEM was not designed for categorical variables (Lefcheck, 2024). Second, in the mixed model

analysis, the number of pup points visible was included for the nursing latency model as a covariate to account for the influence of the number of tracked points on pup movement and nursing latency. However, because path analysis considers all models simultaneously and we expect that the effect of the number of pup points tracked on nursing latency is indirectly through pup movement, piecewiseSEM models were run with and without the number of pup points tracked as a predictor for nursing latency. In all cases, the model without the number of tracked pup points as predictor of nursing latency had the lower AIC value. Finally, the piecewise model for P9 data had a Fisher's *C* statistic test *p* value $< .05$, indicating poor model fit, so alternative models were run and the model with a Fisher's *C p* value $> .05$ and the lowest AIC value was selected.

Principal component analysis (PCA) was conducted on home cage maternal behavior data across all days. PCA was also conducted on 30 pup measures including P6 and P12 USVs (total calls, average call length, sum duration of calls), pup body weight, serum testosterone, and average body temperature for each day when the dam was on nest and off nest. Average pup temperature during licking and grooming and nursing was not included due to a high number of missing data for those measures. Dams/litters with >50 % missing data were removed from the analysis. Remaining missing values were imputed using k nearest neighbors with the knnImputation function of the DMwR package in R. PCA was run using the prcomp function in R. Group differences in principal components scores were evaluated with 2 × 2 ANOVAs with P0–6 condition and P6–13 condition for all components that explained >5 % of variance. No PCs were significantly different by pup sex for any pup data PCs, so the ANOVA tests included data from both sexes.

Serum samples for testosterone analyses were run on two plates and plate was included as a random factor in the mixed model. The initial mixed model included both sexes and follow-up analyses examined the effects of LBN on males and females in separate models.

3. Results

3.1. Body weight

Pup body weight on P3 and P6 are shown in Table 1 and pup body weight on P12 and P13 are shown in Table 2. On P3, male pups were heavier than female pups ($t = 3.08$, $p = .005$) with no effect of LBN. On P6, LBN pups weighed less than Control pups ($t = -4.75$, $p < .001$; **Supplemental Fig. 3**A) and males weighed more than females ($t = 3.96$, $p < .001$). On P12, there was a significant sex difference ($t = 3.88$, $p < .001$). On P13, the P0–6 LBN by P6–13 LBN interaction reached significance ($t = -2.16$, $p = .032$; **Supplemental Fig. 3**B) and males were significantly heavier than females ($t = 3.83$, $p < .001$), with no main effects of P6–13 LBN or P0–6 LBN exposure.

3.2. Pup core temperature

Home cage pup body temperature on P3 when the dam was off the nest was significantly positively associated with temperature in the housing room ($\beta = 0.49$, SE = 0.15, $t = 1.05$, $p < .001$) and was lower in the LBN pups ($\beta = -0.81$, SE = 0.34, $t = -2.37$, $p = .030$; Fig. 2A). P3 temperature was not significantly affected by pup sex (male; $\beta = -0.08$, $t = -0.57$, $p = .571$) or pup body weight ($\beta = 0.14$, SE =0.13, $t = 1.05$, $p = .298$; Fig. 2B).

On P6–13, pup body temperature continued to increase with age ($\beta = 0.08$, SE = 0.01, $t = 7.42$, $p < .001$) and P6–13 LBN exposure reduced pup body temperature ($\beta = -0.75$, SE = 0.17, $t = -4.54$, $p < .001$) but there was no effect of pup P0–6 LBN experience ($\beta = -0.05$, SE = 0.11, $t = -0.44$, $p = .661$; Fig. 2C). Dam nest attendance in older pups trended toward reducing pup temperature ($\beta = -0.09$, SE = 0.05, $t = -1.91$, $p = .056$) while pup temperature increased with active nursing ($\beta = 0.61$, SE = 0.16, $t = 3.86$, $p < .001$) and passive nursing ($\beta = 0.83$, SE = 0.16, $t = 2.22$, $p < .001$) but was not changed by licking and grooming. There were also several significant interactions: pup age and concurrent LBN ($\beta = 0.06$, SE = 0.01, $t = 4.43$, $p < .001$), pup age and active nursing ($\beta = -0.04$, SE = 0.02, $t = -2.41$, $p = .016$), concurrent LBN and active nursing ($\beta = 0.48$, SE = 0.21, $t = 2.24$, $p = .025$), a trend for the interaction between pup age and passive nursing ($\beta = -0.07$, SE = 0.04, $t = -1.88$, $p = .060$), and three way interaction for pup age, concurrent LBN, and active nursing ($\beta = -0.07$, SE = 2.19, $t = -3.30$, $p < .001$) such that at older ages, active nursing reduced body temperature for pups currently experiencing LBN.

Table 1
Pup body weight on P3 and P6. Effects of condition on pup body weight. Values are mean (standard deviation) in grams.

Postnatal day	Control male	LBN male	Control female	LBN female	Effect	Beta	t value	p value
3	9.82 (0.26)	9.91 (0.32)	9.37 (0.26)	9.35 (0.27)	LBN	0.03	0.09	0.930
					Sex (male)	0.51	3.08	0.005
6	15.06 (0.16)	14.17 (0.20)	14.24 (0.18)	13.61 (0.22)	LBN	−0.85	−4.75	<0.001
					Sex (male)	0.70	3.96	<0.001

Table 2
Pup body weight on P12 and P13. Effects of condition on pup body weight. Values are mean (standard deviation) in grams.

Postnatal day	Control-Control male	LBN-Control male	Control-LBN male	LBN-LBN male	Control-Control female	LBN-Control female	Control-LBN female	LBN-LBN female	Effect	Beta	t value	p value
12	26.35 (0.51)	25.71 (0.71)	26.60 (0.36)	24.70 (0.53)	25.30 (0.34)	24.50 (0.63)	24.84 (0.56)	23.54 (0.43)	LBN P0–6	−0.77	−1.66	0.098
									LBN P6–13	0.01	0.03	0.975
									Sex (male)	1.25	3.88	<0.001
									LBN P0–6 X LBN P6–13	−1.32	−1.96	0.051
13	27.40 (0.46)	27.47 (0.69)	26.72 (0.56)	25.70 (0.43)	26.15 (0.48)	25.87 (0.63)	26.11 (0.43)	23.98 (0.45)	LBN P0–6	−0.72	−1.148	0.141
									LBN P6–13	−0.55	−1.10	0.272
									Sex (male)	1.25	3.83	<0.001
									LBN P0–6 X LBN P6–13	−1.54	−2.16	0.032

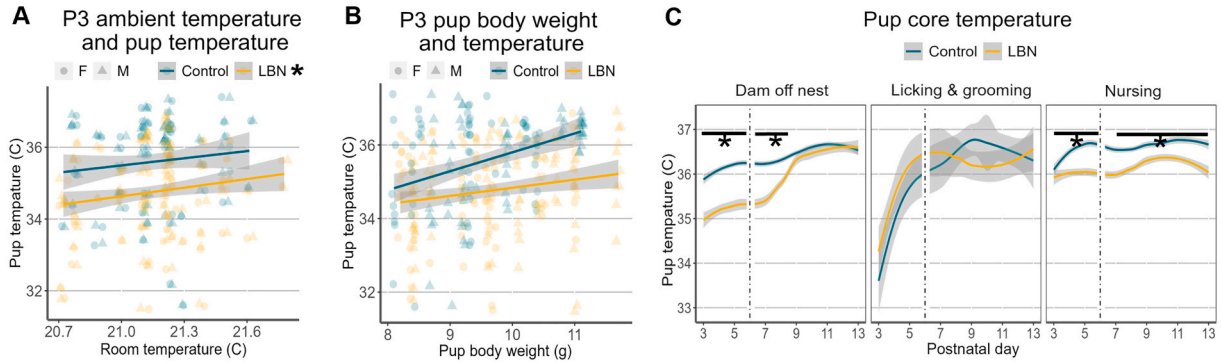

Fig. 2. Pup home cage core body temperature. A. When the dam was off the nest, P3 pup body temperature was positively associated with temperature in the housing room and LBN pups had significantly lower body temperature than Control pups. B. Pup body weight was positively associated with pup temperature, but LBN pups did not benefit from the increase in body weight to the same degree as Control pups. C. Pup temperature increased with age and was lower in the LBN group. LBN pup temperature was comparable to Control pups when the dam was off the nest starting at P9. Licking and grooming reduced pup temperature on P3–6 and nursing (active and passive nursing shown combined) significantly increased pup temperature. LBN reduced pup temperature at older ages during nursing. * Main effect of pup concurrent condition. Bars indicate days when groups differ.

3.3. Home cage behavior

All results for home cage nest attendance, licking and grooming, nursing, and pup movement models are shown in **Supplemental Table 3** (P1 and P3) and **Supplemental Table 4** (P7 and P9). Data for each behavior during the light and dark phase were also analyzed separately, but this did not change the results, so only overall data are presented.

3.3.1. Effects of P0–6 LBN on maternal and pup behavior on P1 and P3

Dams in LBN spent more time on the nest ($t = 8.40$, $p < .001$), more time nursing ($t = 3.85$, $p < .001$), and more time licking and grooming pups ($t = 4.15$, $p < .001$) than dams in the Control condition on P1 and P3 (Fig. 2A). There was no difference in bout duration for any of these behaviors, but LBN dams had shorter intervals between nest attendance ($t = -6.61$, $p < .001$), nursing ($t = -3.21$, $p = .002$) and licking and grooming ($t = 4.41$, $p < .001$). Compared to when the dam was off the nest, pup movement increased when the dam entered the nest ($\beta = 0.01$, SE = 0.01, $t = 42.20$, $p < .001$) and decreased during nursing ($\beta = -0.08$, SE = 0.01, $t = -361.45$, $p < .001$). Compared to Control pups, LBN increased pup movement during nursing ($t = 4.56$, $p < .001$), but did not affect pup movement when the dam was off the nest or on the nest and not nursing.

3.3.2. Effects of P6–13 LBN on maternal and pup behavior on P7 and P9

Dams in LBN spent more time on the nest ($t = 5.34$, $p < .001$) and more time licking and grooming pups ($t = 3.84$, $p < .001$) than dams in the Control condition on P7 and P9 (Fig. 2A). There was no difference in bout duration for nest attendance and nursing, but LBN dams had longer licking and grooming bouts ($t = 2.72$, $p < .001$). LBN dams had shorter intervals between nest attendance ($t = -3.38$, $p = .002$) and licking and grooming ($t = -3.54$, $p = .001$) bouts. Compared to when the dam was off the nest, pup movement increased when the dam entered the nest ($\beta = 0.05$, SE = 0.01, $t = 170.20$, $p < .001$) and decreased during nursing ($\beta = -0.07$, SE = 0.01, $t = -523.25$, $p < .001$). There were no differences between LBN and Control pup movement when the dam was off nest, nursing, or on nest but not nursing.

3.3.3. Effects of pup P0–6 LBN on maternal and pup behavior on P7 and P9

There was a trend for an increase in total time spent nursing on P7 and P9 for dams with pups exposed to LBN on P0–6 ($t = 1.72$, $p = .09$: Fig. 4C). There was a significant main effect for dams with pups exposed to LBN on P0–6 to have shorter licking and grooming bouts on P7 and P9 compared to dams with pups exposed to Control conditions on P0–6 ($t = -2.24$, $p = .025$; Fig. 4B) with no effect on total time spent licking and grooming (**Supplemental Table 4**). In addition, bout duration of nest attendance on P7 and P9 was longer in dams that had pups exposed to LBN on P0–6 ($t = 2.03$, $p = .042$; Fig. 4A). There was also a two-way interaction for dam bout duration between pup P0–6 LBN exposure and postnatal day ($t = -2.45$, $p = .014$) and a three-way interaction between postnatal day, pup P0–6 LBN exposure, and pup P6–13 LBN exposure ($t = 2.16$, $p = .031$).

3.3.4. PCA of dam home cage behavior

PCA of all dam home cage behavioral measures showed that PC1, which accounted for 30.23 % of the variance in the data, separated dams by exposure condition ($F(1,31) = 33.873$, $p < .001$; Fig. 3C-D). Top loadings for PC1 included negative loadings of total durations of behavior and positive loadings of bout intervals (Fig. 3E). There were no significant differences by condition for any of the other top seven PCs.

3.3.5. Effects on maternal latency to begin nursing

Latency to begin nursing after coming onto the nest was significantly longer for dams concurrently exposed to LBN on P1 and P3 ($t = 2.56$, $p = .016$) and on P7 and P9 ($t = 2.45$, $p = .014$). An overall model of dam nursing latency incorporating pup movement, postnatal day, and concurrent condition showed that pup movement had the strongest effect on nursing latency ($t = -4.21$, $p < .001$), postnatal day reduced nursing latency ($t = -3.22$, $p = .001$), but concurrent LBN did not significantly affect nursing latency (**Supplemental Table 5**). However, pup movement between nest attendance onset and initiation of nursing was reduced by LBN ($t = -3.52$, $p = .001$).

Latency models for P3 and P9 were also constructed to account for changes in maternal behavior patterns with pup age and to examine effects of pup previous LBN exposure. On P3, pup movement had the strongest effect on nursing latency, with more movement resulting in shorter latencies ($t = -2.89$, $p = .004$). There was also a trend for LBN to increase nursing latency ($t = 1.77$, $p = .079$) and a trend for an interaction of LBN and pup movement on nursing latency ($t = 1.83$, $p = .069$). On P9, P6–13 LBN did not affect pup movement ($t = -0.86$, $p = .39$), but pup P0–6 exposure to LBN increased P9 pup movement during nest attendance prior to nursing ($t = 2.14$, $p = .044$). Similar to P3, pup movement reduced nursing latency ($t = -2.99$, $p = .003$; **Supplemental Table 5**). Neither P6–13 LBN or pup P0–6 LBN exposure had a significant effect on P9 nursing latency.

Path analysis of nursing latency results supported the mixed model results in terms of significant association and direction of effects (**Supplemental Table 6**). The path analysis of data from all days showed that

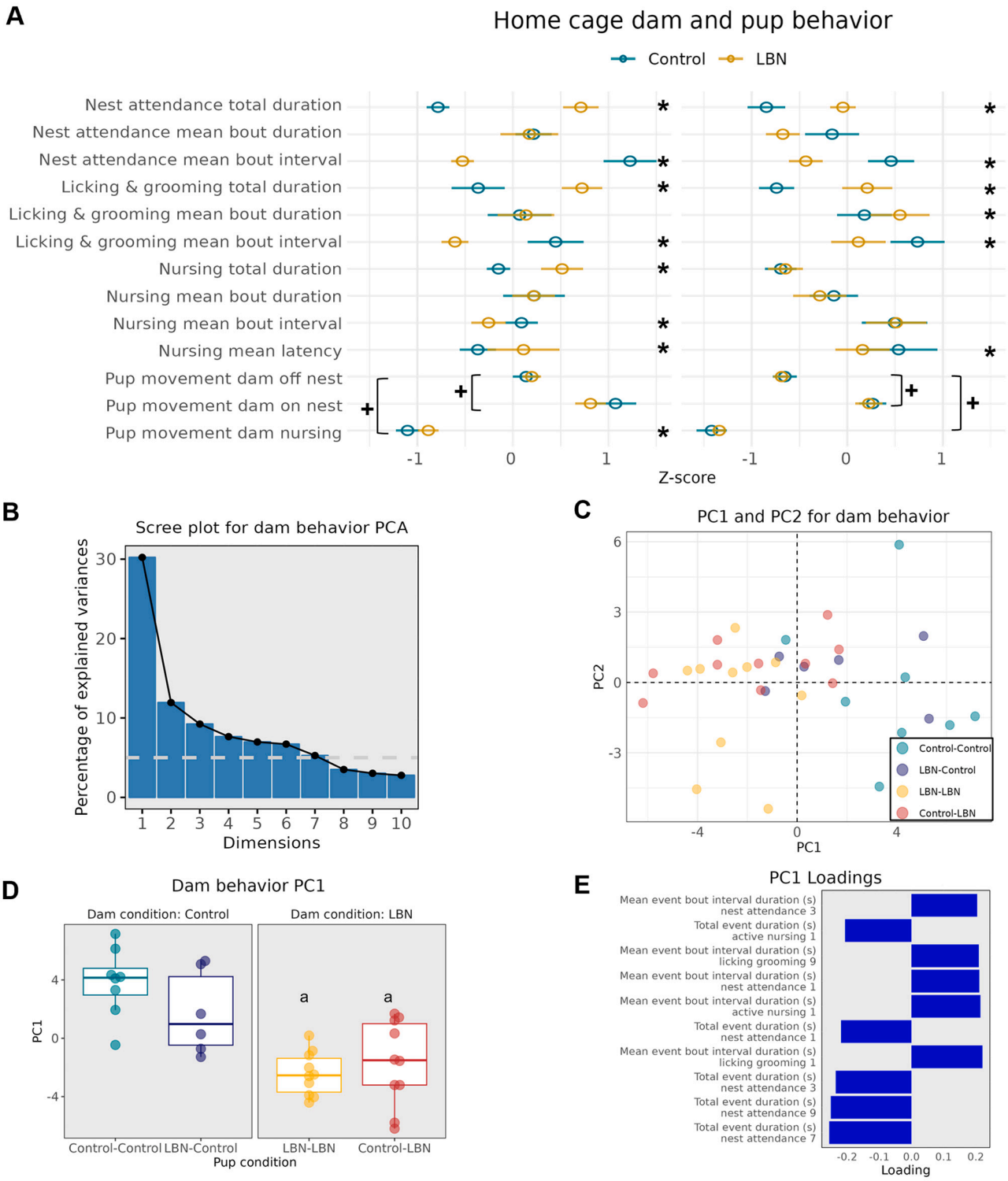

Fig. 3. Effects of concurrent LBN on home cage behavior. A) Concurrent LBN affected several home cage behaviors. Each maternal behavior measure was z scored across all postnatal days. Pup movement z scores were calculated including all pup movement measures. B) Scree plot of principal component analysis of dam behavior across all days. Principal components (PC) 1–7 each accounted for >5 % of the data. C) PC1 and PC2 plot for dam behavior data. PC1 accounted for 30.23 % of variance in the data and separated dam condition. Rather than distinct clusters, LBN and Control dams are distributed along a continuum. D) PC1 scores by dam and pup groups. Dams in the LBN condition had significantly lower scores of PC1, regardless of pup previous condition. E) Top loadings for PC1. Total event durations of maternal behaviors loaded negatively on PC1 and mean bout intervals loaded positively on PC1. [a]effect of concurrent dam exposure to LBN $p < .05$.

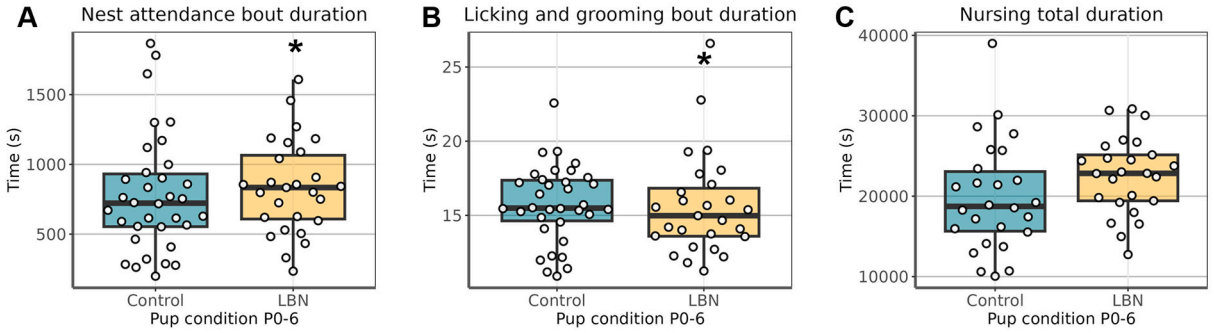

Fig. 4. Effects of pup exposure to LBN on P0–6 on maternal behavior on P7 and P9. A) Dams with pups exposed to LBN on P0–6 had longer nest attendance bout durations on P7 and P9. B) Dams with pups exposed to LBN on P0–6 had shorter licking and grooming bouts compared to dams with pups exposed to the Control condition on P0–6. C) Total duration of nursing was longer on P7 and P9 for dams with pups in LBN on P0–6, but this did not reach significance ($p = .09$). * Main effect of pup exposure to LBN on P0–6.

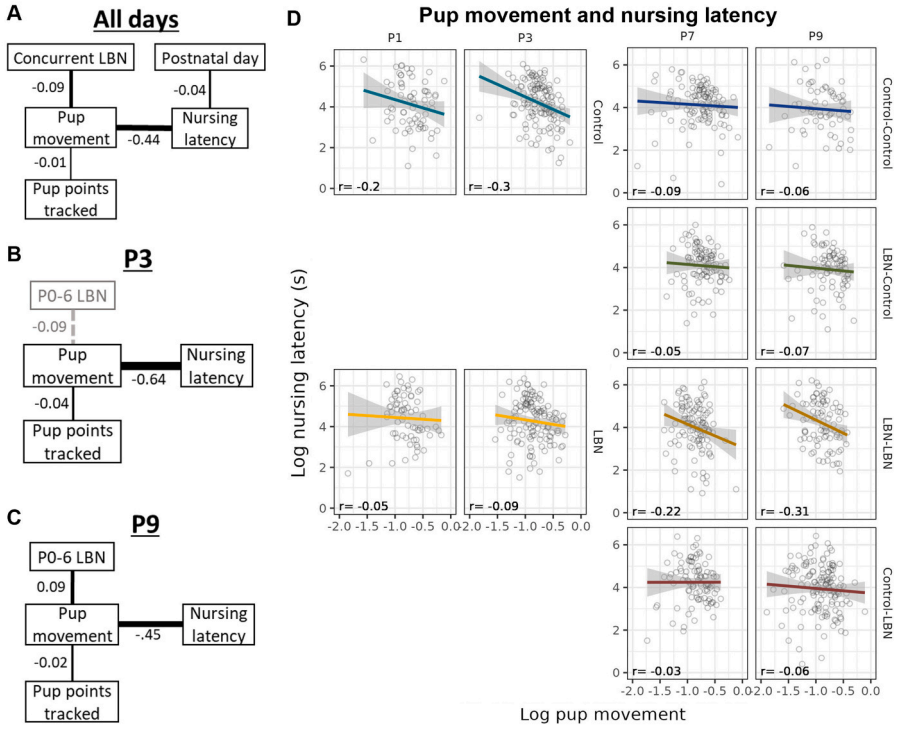

Fig. 5. Effects on nursing latency. Path diagrams from structural equation modeling analysis of the relationships between pup movement, LBN, postnatal day, tracked pup points, and nursing latency during home cage recordings for A) all days (P1, P3, P7, P9), B) P3 only, and C) P9 only. Estimates and line thickness indicate the strength of effect. Solid lines indicate significant effects ($p < .05$) and the grey dashed line indicates a trend-level relationship ($p = .056$). D) Post-hoc correlations between pup movement and nursing latency stratified by postnatal day and condition. The negative relationship between pup movement and nursing latency weakens across postnatal days for all groups except LBN-LBN.

pup movement had the strongest association with nursing latency and an indirect path between Concurrent LBN exposure and Nursing latency where LBN exposure reduced pup movement (AIC = 3771). There was a direct path between postnatal day and nursing latency, but no effect of postnatal day on pup movement (Fig. 5A). In path analysis of P3 data, the magnitude of effect of pup movement to reduce nursing latency was stronger, but the association between P0–6 LBN exposure and pup movement was no longer significant ($p = .056$; AIC = 822; (Fig. 5B). In the path analysis of P9 data, the effect of P0–6 LBN exposure was opposite of that in the overall model; P0–6 LBN exposure increased, rather than reduced, pup movement (AIC = 1242; Fig. 5C). More pup movement on P9 predicted a shorter nursing latency. In all path analyses, the mean number of pup points tracked had a significant negative effect on the pup movement measure.

Follow-up analysis examined correlations between nursing latency and pup movement stratified across postnatal day and groups (Fig. 5D). For dams and pups in Control conditions, the negative relationship between pup movement and nursing latency weakens as pups age. LBN-Control and Control-LBN groups show similar relationships between pup movement and nursing latency on P7 and P9 as the Control-Control group. However, the negative relationship between pup movement and nursing latency remains evident on P7 and P9 for the LBN-LBN group.

3.4. *Pup ultrasonic vocalizations*

P6 USV results are shown in **Supplemental Table 7**. Pup weight, time separated from the dam before testing, and time of day of test (testing order) were included as covariates for USV analysis. The models

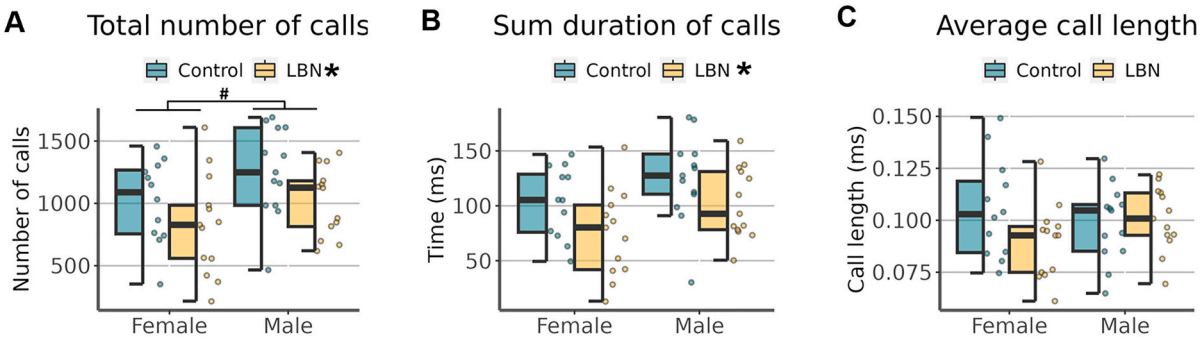

Fig. 6. P6 USV emissions. A) Males emitted more calls than female pups and LBN pups emitted fewer calls than Control pups. B) The total duration of all calls was shorter in LBN pups compared to Control pups. C) LBN pups had a trend for shorter call length compared to Control pups and D) a non-significant reduction in latency to emit the first biphonation call. * effect of LBN $p < .05$, # effect of pup sex $p < .05$.

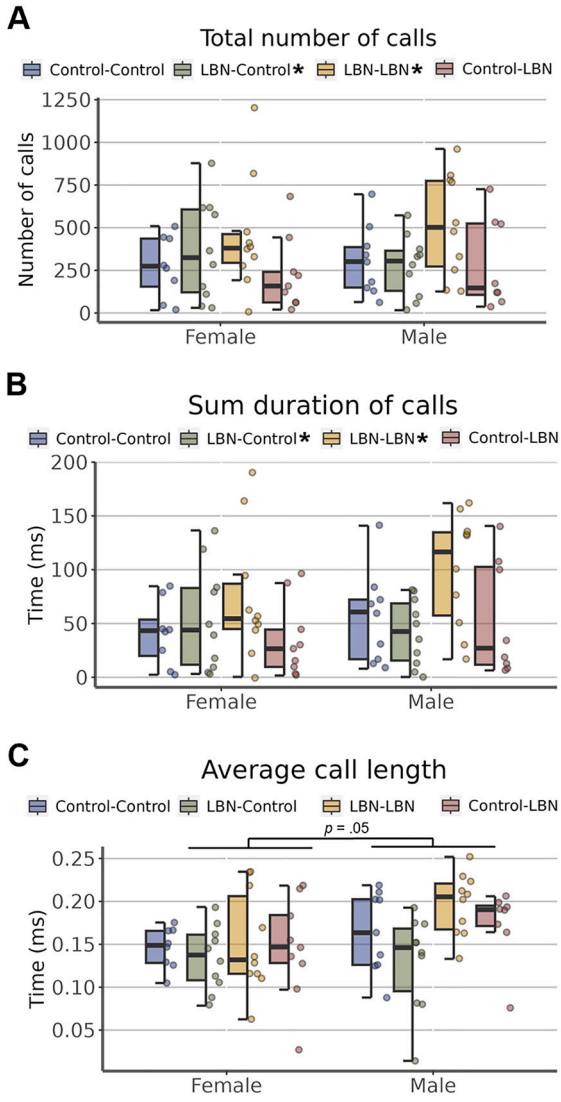

Fig. 7. P12 USV emissions. A) Pup LBN exposure on P0–6 increased the total number of calls produced. B) Pup LBN exposure on P0–6 increased the total duration of calls and concurrent LBN exposure trended toward increasing the duration of all calls. C) Concurrent LBN trended toward increasing the average calls length. Calls from male pups were marginally longer on average than calls from female pups. * significant effect of P0–6 LBN exposure $p < .05$.

with the lowest AIC/BIC values included these factors for P6 data, but not for P12 data.

On P6, male pups emitted more calls than female pups ($t = 3.25$, $p = .003$) and LBN pups emitted fewer calls than Control pups ($t = -2.37$, $p = .028$; Fig. 6A). The total duration of calls across the recording period was also shorter for LBN pups ($t = -2.80$, $p = .008$; Fig. 6B). There was no effect of LBN on average call length (Fig. 6C). The proportion of calls categorized as "short" was lower for males than female ($t = -2.06$, $p = .050$) and there was a significant sex by condition interaction such that LBN male pups had a smaller proportion of very short calls compared to other groups ($t = -2.16$, $p = .040$). No other differences in call type were found on P6.

P12 USV results are shown in **Supplemental Table 8**. Pups exposed to LBN on P0–6 had more total calls during P12 USV testing compared to pups that experienced Control conditions on P0–6 ($t = 2.29$, $p = .025$; Fig. 7A). Male pups had a lower proportion of short calls ($t = -1.88$, $p = .068$) and fewer very short calls ($t = -3.06$, $p = .005$). P6–13 LBN also reduced the proportion of very short calls ($t = -2.13$, $p = .042$).

3.5. *Pup testosterone*

As expected, male pups had higher serum testosterone than female pups on P13 ($\beta = 1.57$, SE = 0.70, $t = 3.03$, $p = .004$). Pup exposure to LBN on P6–13 significantly increased testosterone levels ($\beta = 1.37$, SE = 0.68, $t = 2.01$, $p = .049$), with no effect of pup exposure to LBN on P0–6 (Fig. 8A). Follow-up analysis stratified by sex revealed the effect of P6–13 LBN exposure was driven by females ($\beta = 1.42$, SE = 0.55, $t = 2.61$, $p = .013$) and was not significant in males alone ($\beta = 0.48$, SE = 0.82, $t = 0.58$, $p = .566$). Pup serum testosterone levels on P13 were significantly positively associated with the total duration of licking and grooming on P7 ($r = 0.26$, $p = .038$) and P9 ($r = 0.38$, $p = .003$; Fig. 8B). When analyzed separately, the relationship between testosterone and licking and grooming total duration for females was stronger than for males on P9 (females: $r = 0.40$, $p = .030$; males: $r = 0.36$, $p = .068$) and P7 (females: $r = 0.28$, $p = .012$; males: $r = 0.23$, $p = .238$). Testosterone was also negatively associated with P9 licking and grooming bout interval ($r = -0.38$, $p = .010$; females: $r = -0.32$, $p = .091$; males: $r = -0.40$, $p = .044$; Fig. 8C). Testosterone was not significantly associated with other measures of nursing, licking or grooming, or nest attendance on P7 or P9 (all $p > .05$).

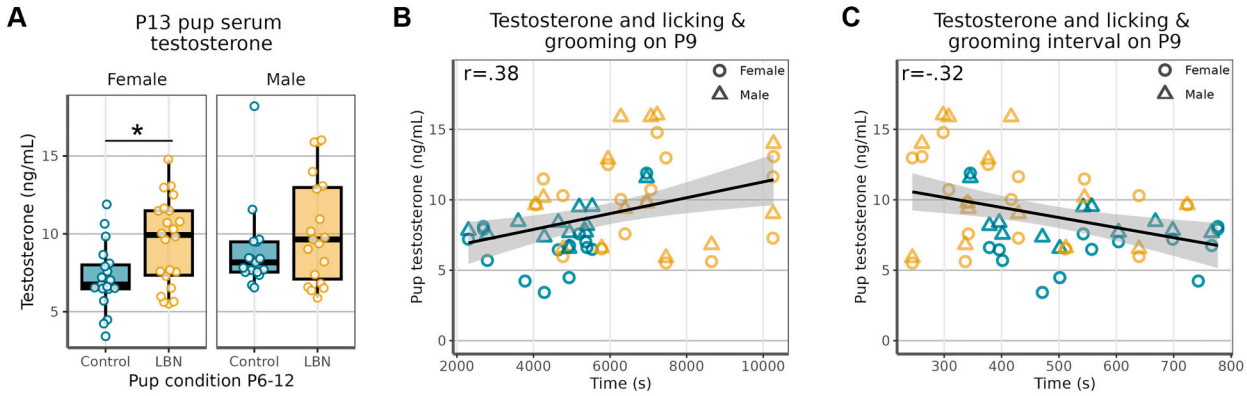

Fig. 8. Pup testosterone. A) Concurrent LBN exposure on P6–13 increased pup testosterone. When analyzed separately, the effect of LBN was only significant for females. B) Testosterone levels were significantly positively correlated with maternal licking and grooming and C) negatively correlated with licking and grooming bout intervals on P9. * $p < .05$.

3.6. Pup measures PCA

Seven PCs from PCA of pup measures explained >5 % of variance in the data in pup body temperature, pup USVs, pup weight, and pup serum testosterone (**Supplemental Fig. 4A**). None of the top seven PC showed separation by pup sex, but PC2 ($F(1,60) = 6.37$, $p = .014$) and PC5 ($F(1,61) = 4.72$, $p = .034$) scores were significantly different between pups with and without P6–13 LBN exposure (**Supplemental Fig. 4B—C**). PC5 scores were also significantly different between pups exposed to P0–6 LBN vs Control conditions ($F(1,61) = 4.16$, $p = .046$; **Supplemental Fig. 4C**). Top loadings for PC2 and PC5 included pup weight and pup body temperature when the dam was on and off the nest (**Supplemental Fig. 4D-E**). In addition, significant P0–6 LBN x P6–13 LBN exposure interactions were observed for PC4 ($F(1,60) = 7.79$, $p = .007$) and PC7 ($F(1,61) = 5.20$, $p = .026$).

4. Discussion

Early life environments can alter developmental trajectories of offspring through alterations in bidirectional parent-offspring interactions. While LBN has been demonstrated to induce maternal stress, the bidirectional interplay between mothers and offspring in this paradigm has not previously been explored and may be a critical mechanism to consider in the immediate and long-term effects of this early rearing environment. In this study, concurrent LBN generally increased pup-directed maternal behavior in an age dependent manner, shifting maternal phenotypes along a spectrum. As predicted, LBN also altered several pup cues known to affect maternal behavior including reducing home cage pup core body temperature, reducing pup weight, altering pup vocalizations, and elevating circulating testosterone in pups. Furthermore, concurrent LBN reduced pup movement between nest attendance onset and the start of nursing, which was negatively related to dam nursing latency and contributed to longer nursing latency in LBN dams. We also demonstrate lasting effects of pup P0–6 LBN exposure that led to longer nest attendance bouts and shorter licking and grooming bouts on P7 and P9.

Neonatal rat pups are endothermic and have limited physiological thermoregulatory capabilities, so they rely on peer huddling and warmth from the dam to maintain optimal body temperature for growth and development (Shelton and Alberts, 2018). Aligned with our predictions, LBN reduced pup body temperature when the dam was off the nest until P9, consistent with a cooler nest microclimate due less cohesive pup huddling and lack of bedding insulation (Lapp et al., 2020b; Fig. 2C). A reduction of pup temperature with LBN was similarly demonstrated in a recent study showing that P10 rectal temperature was lower in LBN Sprague-Dawley pups at the conclusion of a P2–9 LBN exposure (Shupe and Clinton, 2021). The use of implanted BioTherm tags in the current study permitted a thorough analysis of pup body temperature in the home cage to capture age-related changes in thermal effects across the experiment and incorporate dam behavior in the analysis. The increase in pup temperature when the dam is off nest as pups age, particularly evident for the LBN group, aligns with the ontogeny of pup physiological and behavioral thermal regulation during the first two weeks of life, with onset of shivering, piloerection, vasoconstriction, and insulative fur beginning after P6 (Shelton and Alberts, 2018). The effect of dam nest attendance on pup temperature was dependent on the specific dam behavior, where licking and grooming lowered pup body temperature and nursing increased pup body temperature. Although the dam is a source of warmth for the pups, non-nursing periods of nest attendance (e.g. licking and grooming at nest attendance onset) involves limited dam-pup focal contact and rearranging the pups, disrupting huddling behavior and reducing pup-pup contacts before consistent, prolonged dam-pup thermal-tactile contact is established during nursing. The lower body temperature of LBN pups during nursing at older ages may be explained by pups being relatively spread-out during nursing with LBN pups lacking the insulation from bedding material that is available to Control pups to help maintain warmth. Chronic lower body temperature in LBN pups may be one signal increasing the time LBN dams spend on the nest, as has previously been shown with pups cooled at 22.5C (Leon et al., 1978). In a model of early deprivation, P1–14 exposure to 21C for 4 h per day reduced pup body weight compared to pups exposed to 32C (Rüedi-Bettschen et al., 2004). Chronic cold exposure in our study may similarly be responsible for reducing body weight of LBN pups.

Pup vocalizations are distal cues for dams that can signal affective state, physiological state, and proximity to the dam. The reduction of LBN number of calls, duration of all calls, and average length of each call on P6 do not align with the increase in pup-directed maternal behavior in LBN dams on P1 and P3, perhaps suggesting that vocalizations are not a primary cue driving home cage nest attendance, nursing, licking and grooming (Fig. 6). Indeed, the utility of pup vocalizations in eliciting maternal attention has primarily been demonstrated in the context of pup displacement from the nest (e.g. during the pup retrieval task), which is relatively rare under undisturbed conditions, or as a consequence of rough handling by the LBN dam (Boulanger-Bertolus et al., 2017; Branchi et al., 2001; Ihnat et al., 1995; White et al., 1992; Wöhr et al., 2010). The effects of pup LBN experience P0–6 on P12 vocalizations were opposite to those on P6 (Fig. 7) and although the main effect of P0–6 LBN was significant, the group means of the LBN-Control and Control-Control groups were similar for the total number of calls and call durations, suggesting this effect was driven primarily by the LBN-LBN group. P6–13 LBN also increased the total call time and average call

duration on P12, although the effects did not reach significance. These data align well with other work showing fewer calls emitted by female LBN pups on P6-P10 and more calls emitted on P13–19 under thermal-neutral recording conditions in an experiment using a wire platform version of LBN (Granata et al., 2021). Central and peripheral developmental changes between P6 and P12, such as improvement of thermal regulation strategies and development of the hypothalamic-pituitary-adrenal axis, may contribute to differential effects of LBN on vocalizations at these different developmental stages.

Circulating testosterone is a hormonal cue that regulates the amount of anogenital licking and grooming a pup receives from the dam (Moore, 1982). We found elevated serum testosterone with P6–13 LBN on P13 (Fig. 8A). This effect was driven by females, although serum LBN pup testosterone levels were more variable than Control pup levels in both sexes. The impact of postnatal environmental factors on female serum testosterone has not been extensively explored, although our findings align with a study showing elevated testosterone in female fetuses at gestational day 18 following repeated daily restraint stress during gestation in CF-1 mice (Vom Saal et al., 1990) and a report that neonatal cold exposure has the capacity to alter serum testosterone in male pups (Matuszcyk et al., 1990). The source of this testosterone increase in females should be explored in future studies. Although other pup-directed behaviors were also increased with P6–13 LBN, the associations between testosterone and dam behavior were specific to licking and grooming. These data are consistent with the notion that testosterone induces androgen-dependent changes in preputial chemosignals that are released through pup urine and sensed by the dam to promote licking (Moore, 1992). Although there was no effect of P0–6 LBN exposure on P13 testosterone, this hormone was only measured on P13. It is possible that LBN increased testosterone in pups exposed P0–6, but the effects were no longer evident at P13.

Coordination of dam and pup behaviors is vital for the progression of development. In this study, pup movement levels reflected dam movement when the dam was interacting with pups: pups moved more when the dam was on the nest engaging in behaviors with high levels of movement (e.g. licking and grooming) and moved less during nursing when the dam is also relatively immobile. Pup movement was also the strongest predictor of nursing latency across all days (Fig. 5A-C). The negative association between pup movement and nursing latency is highly consistent with previous work showing pup movement against the dam's ventrum promotes active nursing, with less ventral stimulation (i.e. fewer pups or smaller pups) leading to a longer time to enter a quiescent nursing posture and eject milk (Stern and Johnson, 1990). Notably, pup movement in this period was calculated using movement of all pups visible in the recording, which may or may not be in contact with the dam. More accurately capturing tactile stimulation specifically to the dam's ventrum would improve this measure. Interestingly, the negative relationship between pup movement and nursing latency weakened with pup age in all groups except for LBN-LBN rearing condition and was weaker in the LBN group compared to the Control group at P1 and P3. It is currently unknown how LBN modifies the influence of pup movement on nursing onset.

We used a cross over design to examine lasting effects of pup prior experience on maternal behavior. Overall, pup P0–6 exposure to LBN was less impactful on P7 and P9 maternal behavior than P6–13 LBN exposure (Fig. 4). Nevertheless, our data support the idea that pup experiences can affect some pup-directed maternal behavior in subsequent days. The specific maternal behaviors that are affected by pup previous experience may be sensitive to specific pup cues affected by environmental conditions over a longer period of time. Cues that change more slowly, like body weight and body size, may be more likely to affect maternal behavior in the future compared those that change rapidly in response to the environment, such as body temperature. Lasting effects of P0–6 pup exposure to LBN were also detected for the number of vocalizations and sum duration of all pup calls on P12, showing persistence of LBN effects on pup behavior beyond the exposure period.

The effects of limited bedding on maternal behavior have been extensively explored and appear to vary depending on the specific LBN methodologies including developmental timing, use of wire mesh, species and strain of animals, and bedding type (Orso et al., 2019; Walker et al., 2017). Notably, there is also considerable variation in how maternal behavior is measured and what behaviors are measured. Among methods using the scarcity-adversity protocol (without wire mesh platform), most studies have used 30 min – 1 h focal observations (e.g. Gifford et al., 2023; Lapp et al., 2020a; Lapp et al., 2020b; Moriceau et al., 2009; Raineki et al., 2015; Rincón-Cortés and Grace, 2022; Roth and Sullivan, 2005) or snapshot observations where presence or absence of behavior are recorded a 3–5 min intervals (e.g. Fuentes et al., 2014; Shupe and Clinton, 2021) for one or more observation periods per day. Our automated approach provides a rich home cage data set for four 24 h periods to capture developmental changes in behaviors as well as clear increases in several pup-directed maternal behaviors for LBN dams (**Supplemental Tables 2 and 3**). Although the effect of LBN on quantity of pup-directed maternal behaviors are mixed, our home cage behavior findings agree with other reports of increased nest attendance and nursing with LBN (Eck et al., 2020; Fuentes et al., 2014; Lapp et al., 2020a; Shupe and Clinton, 2021). Use of the AMBER pipeline provided additional insights into the responsiveness of the dam to pup behavior (movement) through pup tracking and longer observation periods provided more detailed information on bout lengths and bout intervals of dam behavior without potential of observer bias.

LBN effects on maternal behavior also changed across postnatal day. The influence of concurrent LBN on maternal behavior was more apparent in P1 and P3 behavioral measures with group differences shrinking or disappearing in the P7 and P9 recordings. PCA analysis showed that LBN shifted the behavioral phenotype, but dam/litter phenotype for both groups was distributed along a continuous spectrum rather than as distinct clusters (Fig. 3B). Individual differences in dam response to LBN (e.g. food hoarding behavior) that have been described elsewhere may contribute to this variation, but have yet to be systematically investigated (Lapp and Moore, 2020; Walker et al., 2017).

There are several limitations that should be considered when interpreting results from this study. First, dams remained in the same condition for the duration of the experiment, so it is not possible to disentangle effects of LBN that vary with litter age from potential habituation to the LBN environment over time. Second, the pup movement measure only accounts for movement of pups that are tracked by the pose estimation model in the recording. Pups that are occluded by bedding or the dam, as the majority of pups are during active nursing, could not be measured. The lack of bedding material led to fewer pup occlusions in the LBN condition, especially for older pups, which affects the mean pup movement measure. We accounted for group differences in the number of individual pups tracked and the total number of pup points tracked by creating matched data sets. A limitation of the matching data is that frames omitted from the matched data sets may not be random. For example, frames from LBN recordings with a large number of visible pup points that were excluded from the matched data sets may reflect a behavioral event in which the pups move differently than frames where fewer points are visible. Finally, we believe that post-processing the frame-level behavior annotation data improved the overall quality of the home cage data, but there is a chance that this step could have removed group differences in very short behavior bouts from the data set.

Overall, our findings demonstrate that early environmental exposures, such as limited bedding, have the capacity to change pup cues that regulate maternal behavior. We found that LBN reduced pup body temperature, reduced pup weight gain, and increased serum testosterone, which are all shown to increase maternal care. Most effects on pup cues were acute, occurring only during pup LBN exposure. However, we also demonstrated the capacity for lasting effects of pup prior exposure on dam nursing bout length and licking and grooming bout length using the cross over design. Chronic early life cold exposure slows

maturation and delays developmental milestones (e.g. eye opening, independent feeding; Gerrish et al., 1998). Higher levels of maternal care are also associated with slower offspring development (Cameron et al., 2008; Franks et al., 2015). The increase in pup-directed behaviors with LBN may be a compensatory response to environmental conditions to slow the development of LBN pups that are more energetically taxed than pups provided with abundant bedding. Paradoxically, others have shown that LBN accelerates maturation of specific brain circuits, raising the question of potential trade-offs for central and peripheral development that are dependent on environmental context (Manzano Nieves et al., 2020; Rincón-Cortés and Sullivan, 2014). Future work should consider immediate effects of LBN on pups, the relative compromises in central and peripheral development resulting from LBN, and how the environment may modulate the relationship between pup cues and maternal behavior.

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CRediT authorship contribution statement

Hannah E. Lapp: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Melissa Salazar:** Investigation. **Frances A. Champagne:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2024.105630.

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