Age-dependent impact of early-life stress on glia and synapses
Substrates for increased risk for Alzheimer's disease
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Hallmarks of effective exposure to the limited bedding and nesting model of early-life stress: A preliminary report focusing on maternal care and pup surface temperature

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Abstract

Early-life stress (ES) exposure is associated with negative health outcomes later in life. Animal models are an essential tool to understand the mechanisms behind this early programming, of which limiting bedding and nesting (LBN) materials from postnatal days (PND) 2-9 is a very widely used model. However, there are variations and limitations in implementing this model, necessitating the ability to determine successful and comparable ES exposure across experiments. Unfortunately, while many ES research designs investigate phenotypes in adult mice, only few non-terminal readouts (e.g., body weight gain) can be obtained in pups, making it difficult to estimate level of success at early stages. There is thus a need for additional, reliable, and non-invasive readouts next to body weight measures to estimate effective ES exposure. Among the classical readouts of the LBN model is altered mother-offspring interaction (i.e., maternal care behavior), which can, however, be tedious and subject to scoring variability. Additionally, beyond maternal care, it has been proposed that other elements of the model (e.g., cold exposure) might contribute to the LBN phenotype. However, the role of hypothermia in the LBN model and its associated phenotypes has not been thoroughly addressed up to date.

We aimed in this study to 1) find easy, quick, and reproducibly assessable parameters of maternal care behavior in ES mice, and 2) implement an infrared thermal camera-based method to measure surface temperature in ES pups. We found that control, but not ES, dams progressively spent more time outside of the nest. In addition, we did not detect a difference in dams or pup surface temperature in controls versus ES nests. Our finding of an easier and less intensive maternal care phenotyping can possibly be integrated into standard assessments of effective LBN exposure. This, along with the further readouts obtained using non-invasive measures (e.g., surface temperature, ultrasonic vocalizations, etc.) of ES, should help in improving rigor and reproducibility in the field.
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Introduction

While exposure to early-life stress (ES) is associated with increased risk for later-life cognitive decline and pathophysiology\(^1\text{-}^3\), the mechanisms and neurobiological substrates responsible for such lasting programming remain to be determined. Given the limited causal inferences we can draw from human studies, animal models remain an important component to understand the mechanisms resulting in ES-associated phenotypes\(^4\). However, while ES models are able to recapitulate certain cognitive and metabolic features observed ES-exposed individuals\(^5\), there are inherent variations and limitations in their implementations within and between labs\(^6\), as with all animal models\(^7\). As such, it is important to ensure that each model is comparably induced across experiments.

A very commonly used model to induce ES in rodents is by limiting bedding and nesting (LBN) materials in the cage during the first postnatal week, as pioneered by the Baram lab\(^8,9\). This model leads to alterations in pup body weight gain\(^9\text{-}^15\), corticosterone levels\(^9\), thymus weight\(^10\), and plasma glucose levels\(^12\), and is associated with later life cognitive deficits and alterations in e.g., neurogenesis\(^10,14\), synaptic plasticity\(^16\), and microglial dysfunction\(^17\). However, while this model is widely used to investigate lasting, adult phenotypes after ES, reduced body weight gain is currently the only non-terminal benchmark of effective ES exposure in LBN pups.

To supplement this, much attention has been paid to characterizing maternal behavior as a readout of the LBN model. In fact, due to the increased number of nest exits that LBN dams take\(^9,10\), as well as the increased unpredictability of their behaviors\(^18\text{-}^20\), it is proposed that ES effects in pups are induced by the fragmentation of maternal care. However, while maternal care observations are thus a useful readout to supplement pup body weight gain in assessing ES exposure, these can be logistically difficult and time consuming if nests are staggered in birth dates. These observations are additionally prone to inter-observer differences, making it pertinent to develop simpler, more reliable ways to characterize maternal care.

Moreover, while maternal care quality exerts a strong influence in developmental and later-life outcomes\(^21\), some have also suggested a role for other nest factors, such as temperature\(^22\). Like all biological creatures, mice buffer their core body temperature and maintain homeostasis by staying within a thermoneutral zone\(^23\). Beyond this point (around 30°C in mice), animals activate biochemical mechanisms\(^24\), or make use of environmental resources (e.g. nesting material), to alleviate thermal stress\(^25\). Because the reduction of nesting materials is a key part of the LBN model, it has also been speculated that hypothermia might be another factor contributing to the observed phenotypes after ES. Importantly, previous work from our group found increased adipose tissue browning and UCP1 gene expression (measures of thermogenesis) in the peripheral fat of ES pups\(^26\), further suggesting differences in the thermal profile of ES pups. As such, we wanted to explore the utility of surface temperature measurement, done non-invasively using an infrared thermal camera\(^27,28\), as an additional benchmark of the LBN model.
In this chapter, we present preliminary data from our further characterizations of the LBN model. We report a new parameter of maternal behavior, the proportion of behaviors performed off-nest, which is consistently altered in ES dams. In addition, we measured infrared surface temperature in dams and pups at the start and end of the ES exposure and did not find evidence for alteration of surface body temperature by ES.

Materials and Methods

Animals and experimental design
All observations described in this chapter were done on cohorts of mice from a C57BL/6J background (Envigo, the Netherlands), which were parts of larger studies. Nests were observed during exposure of mice to ES using the limited bedding and nesting (LBN) paradigm from postnatal days (PND) 2-9. Surface temperature was measured in 25 nests (11 CTL, 14 ES), while maternal care was observed in 39 nests (19 CTL, 20 ES). Sixteen nests (7 CTL, 9 ES) were subject to both observations.

All breeding was done in-house, as previously reported. Briefly, after a two-week period of acclimatization in the animal facility, 8–10-week-old virgin female mice were bred with 4–6-week-old male mice. Male mice were taken out after one week, and females were single-housed a week later. We started checking for pups between the first two hours of the light phase (Zeitgeber Time, ZT, 0:00-2:00) 18 days after the start of breeding. Nests found at this time had the previous day denoted as PND 0.

Besides the period when exposed to ES, mice were housed under standard housing conditions, defined as a temperature of 20-22°C, 40-60% humidity, cage enrichment and ad libitum standard chow and water. The mice were kept on a standard 12/12 h light/dark schedule (lights on at 8 a.m.). Experimental procedures were conducted according to the Dutch national law and European Union directives on animal experiments and were approved by the animal welfare committee of the University of Amsterdam.

Early-life stress paradigm
Mice were exposed to ES via the LBN paradigm from PND 2-9, as previously reported. Briefly, nests randomly assigned at PND 2 to CTL or ES condition. CTL litters were housed in standard cage settings (around 100 g sawdust bedding, along with a piece of 5x5 cm nesting material (Technilab-BMI, Someren, the Netherlands). ES litters were housed on a fine-gauge stainless steel mesh on top of 33 g of sawdust, along with 2.5x5 cm nesting material. Only nests with litter sizes of 5-6 were used, with larger litters randomly culled to 6 pups. We only used nests with at least one of each sex per nest. In total, we generated 93 CTL pups (45 male, 48 female) and 111 ES (52 male, 59 female) pups.

Maternal care observation
While we have previously analyzed maternal care behavior used an epoch-based sampling method, we made use here of a more simplified sampling method of scoring which has been explored earlier by other groups. This was done by noting the first observed dam behavior every third minute, for a total of 25 observations per session. We built an ethogram, including 7
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on-nest and 5 off-nest behaviors, to determine which behaviors to score based on previous maternal care observations in our group\(^{10}\), which was adapted into a format similar to those used by other groups\(^{19}\). On-nest behaviors scored include: arched-back feeding, blanket feeding, passive on-nest, pup grooming, nesting, self grooming, and an “other” category for all other behavior. Off-nest behaviors scored include: feeding/drinking, self-grooming, exploration, rest outside, and an “other” category for all other behavior. All nests were observed from PND 2-8 between the first two hours (ZT 12:00-14:00) of the dark phase. A subset of nests were also observed in the middle of the light phase (ZT 5:00-7:00).

An entropy rate was calculated from the pooled observations across 8 days, as described by Molet et al.\(^{18}\), using the markovchain package in R\(^{29}\). In a sub-analysis, all off-nest behaviors were classified as one to measure the unpredictability of maternal behavior at the nest, as performed by Knop et al.\(^{19}\). Additionally, we observed ambiguity in the behavioral classification in the CTL nests, due to the abundance of bedding material. To reduce this uncertainty, we also analyzed dam behaviors by classifying them as performed on- or off-nest. The proportion of time spent outside the nest was then calculated per day and analyzed as a repeated-measures design. To validate this approach, we re-classified an older dataset of maternal care observations obtained using an epoch-based sampling based on the current strategy\(^{10}\).

**Surface temperature measurement via thermal imaging**

To assess if ES treatment leads to thermal stress in our mice, we used FLIR One (3rd gen), a smartphone-compatible thermal imaging camera. The camera acquires a 16-bit infrared heat image and a simultaneous RGB image. We took top-view heat images of the whole cage at P2 and at P9 (around 8-10 images per nest each day), which were analyzed via imageJ (Fig. 1). We used median mean grey value (MGV) of all images per nest for statistical comparisons. Regions of interest (ROIs) for surface temperature measurement in the dams and pups were generated via a mask created from the default automated threshold method.

As each thermal image generated by the FLIR One had a scale bar with minimum and maximum temperatures indicated, the mean grey values (MGV) per thermal image were converted to degrees celsius by creating the following formula:

\[
\frac{\text{MGV}_{\text{Animal}} - \text{MGV}_{\text{Cage}}}{256} \times (\text{Max.} - \text{Min.}) + \text{Min}
\]

This conversion served the purpose of standardizing the scale of the images taken at different days, as our pilot data on a water bath imaged at varying temperatures showed that the camera produces linear measurements with roughly similar slopes but varying intercepts (not shown). Each image was normalized to MGVs from a rectangular empty ROI on the upper part of the cage wall (MGV\(_{\text{Cage}}\)). Temperatures were compared using a Welch-corrected T-test in Graphpad Prism 9.3.1.
Figure 1. Surface temperature analysis workflow.
The FLIR camera provides both a standard image in real-color and a heat image in grayscale. After drawing a region
of interest (ROI) in the real-color image to establish the nest boundaries, ROIs around dams and pup nests within
the nests were established using the AutoThreshold feature in ImageJ. The resulting ROI was then split for dams
and pups, with the mean grey value (MGV) for each measured.

Tissue collection
To assess evidence of thermoregulatory adaptations in ES pups, we dissected and flash-
froze the hypothalamus and inguinal white adipose tissue of a sub-cohort of mice at PND
9. Another sub-cohort of mice was transcardially perfused with saline after i.p. injection of
120mg/kg Euthasol. Their brains and inguinal white adipose tissue were then dissected and
fixed in 4% PFA overnight. Analyses of these tissues are currently on-going.

Results

CTL dams perform more off-nest behaviors in the dark phase from P4 onwards
Early-life stress (ES) pups gained less weight between postnatal days (P) 2-9 compared to
controls (CTL) across the cohorts reported here (Condition: $F_{(1,70)}=54.74, p<0.0001$; Sex:
$F(1,70)=0.0002266, p=0.9880$; Interaction: $F(1,70)=0.04188, p=0.8384$, Fig. 2A), confirming
the earlier reported effects in this LBN model by us and others 9–15.

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Figure 2. Control, but not LBN, dams progressively perform more off-nest behaviors during the dark phase between P2-8. (A) Body weight gain is decreased in early-life stress (ES) nests (A). During the dark phase, control (CTL) dams progressively perform more behaviors off-nest (B) across observation sessions, and (C) in total. (D) Dams in CTL conditions perform more switches between on and off-nest behaviors over time. (E) ES dams have a higher unpredictability of maternal behavior as measured by entropy rate. (F) Re-analyzing previous maternal care data obtained using epoch-based sampling reveals parallel increased duration of time spent off-nest in CTL dams. *, condition effect, p<0.05; &, postnatal day x condition interaction, p<0.05; @, post-hoc test: significantly different from CTL group in the same postnatal day (P).

To analyze ES-induced differences in maternal behavior, we observed our nests from P2-P8, specifically analyzing proportion of on-versus-off nest behaviors during the light and dark phase. While dams spent progressively more time off nest in the light phase, this was not affected by nesting condition (Day: F(6,102) =3.441, p=0.0039; Condition: F(1,17) =1.500, p=0.2374; Interaction: F(6,102) =0.3029, p=0.9341, not shown). In contrast, during the dark phase, only CTL dams performed more behaviors out of the nest during the sampling period (Day: F(4.882,170.1) =5.837, p<0.0001; Condition: F(1,36) =31.21, p<0.0001; Interaction: F(6,209) =3.432, p=0.003, Fig. 2B). Specifically, CTL dams performed more off-nest behaviors than ES dams starting from P4 onwards (Šídák’s multiple comparisons test of CTL vs ES – P2: p=0.2291; P3: p>0.9999; P4: p=0.0407; P5: p=0.0333; P6: p=0.0006; P7: p=0.0003; P8: p<0.0001). This is also seen when summarizing amount of off-nest behaviors across all observation days (t(33.55)=5.586, p<0.0001, Fig. 2C).

We also analyzed dam nest exit and behavioral entropy in our data, given the importance of these parameters in prior analyses of maternal behavior during ES\(^9,10,18\). To approximate nest exit (i.e., fragmentation) behavior, we clustered the on-nest and off-nest behaviors per observation session, noting the frequency by which a behavioral switch (on-to-off or off-to-on) occurs. Doing so, we similarly find that CTL dams transition more between on-off behaviors at later observation sessions (Day: F(5.118,180.0) =1.614, p=0.1570; Condition: F(1,36) =1.552=0.2209; Interaction: F(6,211) =2.523, p=0.0222, Fig. 2D), although these were not
significant in post-hoc tests (not shown). The total number of transitions between on-off behaviors across observation days is not significantly different ($t(34.29)=1.435$, $p=0.1602$, not shown).

Looking at the entropy rate, while unpredictability of maternal behaviors per se was not different in CTL/ES dams ($t(18.03)=0.7128$, $p=0.4851$, not shown), there was a trend towards increased randomness of on-off nest transitions in ES dams ($t(19.92)=2.033$, $p=0.0556$, not shown). In particular, the entropy rate for maternal behaviors is significantly higher in ES dams when all off-nests behaviors are grouped as one ($t(19.87)=3.187$, $p=0.0047$, Fig. 2E), as would be “observed” by the pups. To validate whether our findings (i.e., the increased time spent off-nest by CTL dams) are brought about by the instantaneous sampling method of maternal behavior, we re-analyzed time spent off-nest in a previously published epoch-based analysis of maternal behavior\textsuperscript{10}. In doing so, we also see increased time spent-off nest depending on condition and observation day (Day: $F_{(6,129)}=2.649$, $p=0.0186$; Condition:

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**Figure 3.** No difference in surface temperature of ES-exposed dams and pups at P2 and P9. Nest surface temperatures were measured in dams and pups (A) at postnatal day [P] 2, before placement into control (CTL or early-life stress (ES) conditions, (B) at P2, right after placement into experimental conditions, and (C) at P9. (D-G) Dam and pup surface temperatures were not different across experimental groups at P2, (H-I) nor at P9.
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F_{(1,25)}=4.940, p=0.0355; Interaction: F_{(6,129)}=2.865, p=0.0118, Fig. 2F), especially at P7 (Šídák's multiple comparisons test of CTL vs ES at P7: p=0.0355).

LBN treatment does not alter surface temperature in dams or pups
We took thermal images before and after placing nests into either condition at P2 (Fig. 3A, B, average time difference <5min) and at P9 (Fig. 3C) to measure surface temperature in pups. Dams and pups placed into either conditions had similar surface temperatures before (Dams: t(8.576)=1.053, p=0.3211; Pups: t(5.463)=0.5661, p=0.5938, Fig. 3D, E) and after (Dams: t(8.327)=0.09609, p=0.9257; Pups: t(7.424)=0.1259, p=0.9031, Fig. 3F, G) being placed in condition. Similarly, at P9, there was no effect of ES treatment on surface temperatures in dams (t(21.84)=0.6285, p=0.5362, Fig. 3H) or pups (t(19.8)=1.149, p=0.2643, Fig. 3I).

Discussion
In this chapter, we aimed to identify new features of maternal care behavior during limited bedding and nesting (LBN) conditions, as well as contribute to a long-due discussion on whether the LBN model has a hypothermic component, and whether these potential thermal differences could serve as an additional readout of effective LBN exposure. We found across multiple cohorts that increased proportion of off-nest behaviors during the dark phase between postnatal days (P) 4-8 is a characteristic feature of dams housed in control (CTL) but not early-life stress (ES) conditions. We propose that this relatively simple readout can be a reliable indicator of effective ES exposure that can be obtained in future studies. We also used an infrared thermal camera based method to measure surface temperature during ES exposure and did not find differences in dam and pup surface temperature at the start or end of LBN treatment.

Maternal time off nest as a signature of LBN exposure
We show evidence from measurements taken across multiple cohorts for increased off-nest behavior in CTL, but not ES dams. Among the core phenotypes of the LBN model is the increased number of exits from the nest by ES dams, which is inversely correlated to the availability of nesting materials. While we have also previously described this finding, our current data suggest that CTL dams eventually spend more time off-nest compared to ES dams.

It is important to emphasize the difference in “sampling rates” of maternal behavior scoring as a source of this discrepancy. In the previous reports characterizing maternal behavior during LBN conditions, we and others scored dam behaviors using epoch-based measurements, consisting of scoring the full range of dam behavior within a minute. Several one-minute epochs are observed per session, with observations across rounds summed at each postnatal day. In the current study, scoring was done based on the first behavior observed every third minute (for a total of 25 observations per nest), which is later combined to calculate a measure of entropy. This is an approach recently adapted in the characterization of maternal care in the ES field, as it allows for scalability in performing observations while still capturing the most relevant differences in ES vs CTL dam behavior (i.e., the pattern, rather than the total amount, of performed maternal behaviors).
Despite the improvements of this newer, “instantaneous” method of sampling maternal behavior, we found that the proper classification of the specific maternal behavior was open to inter-observer variability, especially within the well-covered CTL nests. As such, we explored the utility of using on-/off-nest behavioral classifications as a readout of ES exposure. Importantly, we found that this measure, which goes hand-in-hand with other readouts of LBN such as entropy, is consistently different across our experimental groups from P4 onwards. While it is unclear why CTL but not ES dams might perform more off-nest behaviors, one might speculate it to be linked to the increased ultrasonic vocalization in ES pups, as observed in a rat study (Berretta et al., unpublished). In any case, we propose this readout as a quicker way to observe ES effects on maternal behavior, especially compared to epoch-based measurements, that will allow for easier comparison of ES induction in larger cohorts and across labs.

**Surface temperature is not affected by LBN**
Besides maternal care, ES exposure via LBN has been suggested to also manifest its effects by leading to hypothermic stress in the pups. We expected to find evidence for this using thermal measurements, due to the important role of bedding materials (which we limit) in reducing heat loss\(^25\), as well as our previous work showing thermogenic adaptations in the adipose tissue of ES-exposed pups\(^26\). This did not result in differences in surface temperature, in line with another study that measured core temperatures in rat pups exposed to ES using the same LBN model at similar timepoints\(^30\).

While adult mice are able to maintain a stable core temperature in response to deviations in ambient temperatures, pups do not develop this adaptive capacity until PND 7-10\(^31\). Consequently, their body temperatures instead vary with that of the environment. These adaptations occur via shivering (i.e., heat generation through muscle movement) and non-shivering processes (i.e., metabolic adaptations including phenotypic conversion of white adipose tissues [WAT] to resemble brown adipose tissues [BAT])\(^32,33\). We have previously described evidence for the latter, as pups at PND9 were found to have increased BAT stores and increased gene expression of the thermogenic mitochondrial uncoupling protein-1 (Ucp1) in their WAT\(^26\). These phenotypes, in conjunction with the prolonged time spent by ES dams on the nest, might be compensatory adaptations to mitigate the cold stress that they most likely are exposed to. It would be interesting to investigate how the surface temperature varies across postnatal days and the day-night cycle. Further characterization of the brains and adipose tissues of these pups will hopefully shed light on these thermoregulatory compensations, and hint towards pathways (e.g., nutrient sensing, energy metabolism) that might be dysregulated by LBN exposure, and possibly contribute to the effects of ES exposure on later-life cognitive and metabolic profiles.

**Importance of establishing non-invasive hallmark readouts of the LBN model**
Our work here has emphasized the importance of identifying (non-invasive) benchmarks of proper LBN implementation. While maternal care observation is clearly one such vital readout, it also has logistical and methodological limitations.

ES experiments are often done via in-house breeding, to avoid the stress associated with delivery of timed-pregnant females. However, the asynchronous births of these nests can
make complete maternal care observations logistically difficult. The nature of the epoch-based observations adds to this difficulty, given the need to accurately account for the duration of all dam behaviors, their transitions, as well as number of exits over multiple nests. While offline analyses via video recordings could be an alternative, these can be expensive and time-consuming, and thus feasible investments only for research questions that aim to further characterize behavioral differences between CTL and ES dams. Other studies, hoping to use maternal care to validate effective ES exposure, might favor a quicker approach like we performed. Importantly, our proposed method to focus on on/off-nest behaviors also circumvents the limitations of assessing specific behaviors, which we expect to improve scoring consistency.

Additionally, as our establishment of a surface temperature measurement protocol shows, there are new, upcoming avenues to develop non-invasive readouts of the LBN model. Regarding temperature in the LBN model, it remains to be determined whether, regardless of pup and dam temperatures, there are differences in ambient temperatures within CTL and ES cages, as would be expected. As we speculated above, it would also be interesting to measure ultrasonic vocalizations, which can be implemented relatively easily, and see whether ES pups have altered vocalizations, and if this might be linked to the overall increased on-nest behaviors exhibited by ES dams. Lastly, going back to maternal behavior, the technological development of more compact infrared (non-thermal) cameras should also soon allow for continuous recording of maternal (and pup) behavior during LBN exposure. These data can then be relatively quickly analyzed using emerging machine learning based tools.

**Conclusion**

Our data suggest that the proportion of off-nest maternal behavior can be a useful parameter to assess the effectiveness of the LBN model. We also provide evidence that surface temperature is not different in ES-exposed dams and pups. The continued characterization of readouts and phenotypes, especially non-invasive ones, is crucial to ensure reliable and effective LBN exposure, allowing for consistency across cohorts.
References


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