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Influence of housing variables on the development of stress-sensitive behaviors in the rat



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HIGHLIGHTS

- Laboratory bedding material influences developmental programming of rats.
- Bedding material during early life can alter stress sensitive measures in adulthood.
- Corncob bedding during sensitive periods decreases adult measures of anxiety.
- Bedding material during adulthood does not affect adult anxiety-like behavior.

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ABSTRACT

Diverse environments early in mammalian life can have profound influences on the physiology and behavior of developing offspring. Environmental factors can influence offspring development directly or through perturbations in parental care. In the current study, we wished to determine if the influence of a single environmental variable, type of bedding material used in laboratory cages, is capable of altering physiological and behavioral outcomes in offspring. Female rats were housed in cages containing wood pulp or corncob bedding and allowed to mature. These rats, while housed on assigned bedding material, were bred and allowed to give birth. At weaning, male offspring were housed on one of the two bedding conditions and tested later in adulthood on stress-sensitive behavioral measures. Postmortem analysis of glucocorticoid receptor expression and CRH mRNA levels were also measured. Maternal care directed at the pups reared in the two different bedding conditions was also recorded. Rats reared from birth on corncob bedding exhibited decreased anxiety-like behavior, as adults, in both open field and light–dark box tasks compared to wood pulp reared animals. Animals that received similar overall levels of maternal care, regardless of bedding condition, also differed in anxiety-like behaviors as adults, indicating that the bedding condition is capable of altering phenotype independent of maternal care. Despite observed behavioral differences in adult offspring reared in different bedding conditions, no changes in glucocorticoid receptor expression at the level of the hippocampus, frontal cortex, or corticotrophin releasing hormone (CRH) mRNA expression in the hypothalamus were observed between groups. These results highlight the importance of early life housing variables in programming stress-sensitive behaviors in adult offspring.

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1. Introduction

Across mammalian species, early life is a time of heightened susceptibility to environmental input, capable of altering the development of offspring behavior and physiology [1–4]. Environmental input, particularly during periods of heightened neuronal plasticity, can increase neuron numbers, synapses, and dendritic branching, as well as influence neuroendocrine systems such as the hypothalamic–pituitary–adrenal (HPA) or stress axis to further alter animal behavior [5–11]. In the

laboratory, different animal housing conditions at various points in the lifespan of the organism (such as providing more complex/enriched cages, or conversely, deprivation) can also influence stress-axis function and behavior. Many studies, including a seminal paper by Crabbe et al. illustrate how minor changes in standard laboratory environments can abolish or reverse genetic effects of behavior in laboratory mice [12,13]. Providing enriched housing conditions to mice typically housed under standard laboratory housing conditions is sufficient enough to attenuate non-spatial memory impairments in NMDA knockout mice, possibly by increasing synaptogenesis [13,14]. Little is known about which specific features of laboratory housing contribute to changes in rodent phenotypes. One fundamental environmental variable, the type of bedding used in cages, may be a source variation in laboratory tests

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of animal behavior. Rodent bedding materials have been demonstrated to influence stress and immune reactivity profiles [15,16], thermoregulation processes [17,18], vocalizations, [19], body mass [20], as well as liver enzyme levels in laboratory rats and mice [21,22]. Research focusing on corncob bedding, which contains measurable levels of phytoestrogens, reports alterations in slow-wave sleep, suppression of male and female reproductive behavior, acyclicity in female estrus cycles, as well as changes in estrogen receptor alpha expression in regions of the brain implicated in aggression and sexual behavior [23–25]. These studies suggest that housing conditions can fundamentally alter animal behavior and CNS function, results which emphasize the sensitivity of CNS developmental plasticity as well as fundamentally alter conclusions drawn from animal studies.

Environmental manipulations of standard laboratory housing parameters can influence offspring directly as described above, or indirectly, through perturbations in parental care. For example, alterations in early post-natal maternal care in the laboratory rat can program the developing HPA-neuroendocrine pathways and behavioral fearfulness when rodents reach adulthood [26–29]. These effects persist throughout the life of the animal and alter risk for stress-related disease [2,4,30]. Manipulation of the physical environment, including access to nesting sites and bedding, perturbs parental care which subsequently influences neuroendocrine and behavioral phenotypes of developing offspring. For example, rat mothers with restricted access to bedding material during the postpartum period displayed more disorganized/fragmented levels of maternal care than controls [31]. Rat dams themselves, with restricted access to bedding material during the postpartum period, also display an increase in HPA reactivity, more stressful behavioral phenotypes and altered hypothalamic CRH expression suggesting that environmental alterations increase maternal stress. While offspring behavior was not reported [31], other studies in which pups whose mothers were given restricted access to nesting and bedding material had deficits in spatial memory, reduced body mass, and an increase in depressive-like behavior that were accompanied by changes in hippocampal CA1 long term potentiation [5].

Using a simple manipulation of environmental parameters, we wished to investigate if the use of different housing materials during the early life period of the laboratory rat was capable of altering offspring behavior as adults and if observed changes in offspring behavior can be accounted for by alterations in maternal care. We reared Long Evans rats on wood pulp or corncob bedding, assessed maternal care during the early postpartum period and subsequently assessed stress-sensitive measures later in adulthood. We hypothesized that animals raised on wood pulp bedding conditions would differ significantly in anxiety-like behavior as adults than animals raised on the corncob bedding. We predicted that rat dams provided with wood-pulp materials would provide greater levels of maternal care to offspring which, in turn, would result in lower stress-reactivity phenotypes as adults.

2. Methods and materials

2.1. Animals and housing

Female Long Evans rats used in this study were purchased from Charles River Breeding Laboratories (Wilmington, MA). Adolescent female rats were pair housed in standard polypropylene cages (27.8 × 17.5 × 13.0 cm) containing either wood pulp or corncob bedding material (1/8" Purelite Sanitized Corncob Bedding and Tek-Fresh Laboratory Animal Bedding, Harlan, Hayward, CA). Animals were allowed to mature for three months on the assigned bedding material. Females were then mated with male stud animals also purchased from Charles River. Male studs were housed on wood pulp bedding prior to mating. For all animals, temperature was kept constant at 20 ± 2 °C and relative humidity was maintained at 50 ± 5%. Rats were kept on a 12-h light–dark cycle (lights on 0700 h to 1900 h) and allowed access to food (Tekland Global Diet #2918) and tap water

ad libitum. Females were allowed to give birth and maternal behavior was recorded as described below. A single 9.5 in. × 5.5 in. paper towel was provided for nesting material to all groups. At PND 21, male offspring from across litters (n = min 14/group), were weaned and pair housed in either corncob or wood pulp bedding conditions. Housing conditions at weaning were the same as that of the postpartum period. After 12 weeks of housing, animals were assessed on several stress-sensitive behavioral tasks described below. A subset of naïve animals (n = 10) housed on wood pulp bedding were switched to the opposite bedding and behaviorally tested after two weeks. Animals were euthanized and post-mortem markers assessed within 48 h of completing behavioral tasks. Breeding, weaning, and rearing of animals were performed simultaneously rather than sequentially. Housing and care of the rats were carried out in accordance with the standards and practices of the UC Berkeley Animal Care and Use Committee.

2.2. Observations of maternal behavior

Female rats were bred and permitted to give birth (n = 12). Day of birth was marked as postnatal day (PND) 0. Maternal observations were performed beginning on PND 1 and continued until PND 5 [10,11,32]. Each litter was observed for 3 h a day at the following times: 0700–0800 h, 1200–1300 h and 1900–2000 h. During each observation session, litters were observed and behaviors recorded every 1 min (*i.e.* each litter was observed 180 times per day for five days). Behaviors recorded included: mother on/off the nest and maternal licking behaviors directed at self or at pups. A distribution curve was generated by calculating the frequency with which pup-directed maternal licking was observed. Maternal licking was expressed as a percentage of the total number of observations performed for each litter. The mean frequency of maternal licking was calculated for the cohort. Animals were weaned on PND22, and pair housed with same sex littermates as described above.

2.3. Behavior

All animals were tested in two stress-sensitive behavioral tasks as adults: the Open-Field Test and the Light–Dark Box Test. Animals were tested on non-consecutive days.

2.3.1. Open-Field Test

To assess anxiety-like behavior, animals were exposed to an open field (a large circular polypropylene arena 140 cm in diameter, 61 cm in height). Each animal was placed in the open-field for 5 min and subsequent behaviors were recorded. The arena was cleaned between animals. Frequency of crosses between the outer arena (14 cm width) and the interior inner arena (112 cm diameter) and amount of time spent in the inner-arena of the open field was quantified. The behavior of each rat was recorded and analyzed by an experimenter blinded to group conditions. The greater amount of time spent in the inner arena was interpreted as a less anxious phenotype [33–35].

2.3.2. Light–Dark Box Test

Similar to the open field, the light–dark box is used to assess anxious behavior in rodents [33,35]. The light–dark box consists of two contiguous acrylic rectangular arenas (76 × 40 cm) connected by a 10 × 10 cm entrance. One arena, the dark box, is black acrylic and sheltered with a black acrylic cover while the second arena, the light box, is constructed of transparent acrylic and is open and uncovered. Animals were initially placed within the dark chamber and allowed 5 min of open exploration. The behavior of each rat was recorded and analyzed by an experimenter blind to the conditions. Time spent in the light box was quantified and interpreted as a behavioral marker of less anxious behavior [33,35].

2.4. Postmortem neuronal markers

2.4.1. Western blots

Glucocorticoid receptor (GR) protein expression in the frontal cortex and hippocampus was assayed *via* western blot in all animals ($n = 8$ per group). Tissue was dissected immediately after euthanasia and snap frozen in liquid nitrogen. Whole hippocampus was removed and frontal cortex dissection was restricted to infralimbic, prelimbic, and anterior cingulate cortices. Upon assay, tissue was homogenized with motor driven pestle in RIPA buffer solution with 1% protease inhibitor (Calbiochem protease inhibitor cocktail set iii, EDTA free). RIPA buffer contained 50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate at a pH of 8.0 at room temperature. The homogenate was then centrifuged at 14,000 g for 30 min. The supernatant was extracted and total protein concentrations were determined *via* Pierce BCA protein assay kit (Thermo Scientific). Twenty micrograms of protein was then loaded and separated with a 7.5% Tris–SDS polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). Proteins were transferred to PVDF membrane (Amersham Hybond-P; GE Healthcare) and blocked with 5% non-fat milk in $1 \times$ TBS-t (Tris-buffered saline, 0.1% Tween-20, pH 7.6) for 1 h. Membrane was then incubated with polyclonal rabbit anti-GR at 1:2000 (Santa Cruz Biotechnology, #sc-1004) and mouse anti-actin at 1:10,000 (Sigma Aldrich, #A1978) overnight at 4 °C. The signal was detected using horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies at a concentration of 1:5000 (Jackson ImmunoResearch). The signal was subsequently enhanced *via* Western Lightning ECL Kit (PerkinElmer; Waltham, MA) and exposed to autoradiography film for visualization. Western band optical density was determined by gel imaging system (MCID Basic, Version 7.0; Imaging Research Inc.) and normalized with the band optical density value of actin as an internal control.

2.4.2. RT-qPCR

Hypothalamic tissue ($n = 8$ per group) was analyzed *via* RT-qPCR. Specific rat primers for several different mRNAs were designed by blasting the primer sequence against NCBI genomic databases and then checking for specificity. Primers were created by Integrated DNA Technologies. Primers are detailed in the table below:

Gene	Direction	Sequence
CRH	+	5'-GGA GCC GCC CAT CTC TCT-3'
	-	5'-TCC TGT TGC TGT GAG CTT GCT-3'
CRH-1R	+	5'-TCC ACC TCC CTT CAG GAT CA-3'
	-	5'-TGC AGG CCA GAA ACA TTG C-3'
CRH-2R	+	5'-CTG GAA CCT CAT CAC CAC CT-3'
	-	5'-AGG TAG CAG CCT TCC ACA AA-3'
BDNF exon IX	+	5'-GAG AAG AGT GAT GAC CAT CCT-3'
	-	5'-TCA CGT GCT CAA AAG TGT CAG-3'
RPLP	+	5'-ATC TAC TCC GCC CTC ATC CT-3'
	-	5'-GCA GAT GAG GCT TCC AAT GT-3'

CRH, corticotrophin releasing hormone peptide; CRH-1R, corticotrophin releasing hormone receptor 1; CRH-2R, CRH receptor 2; BDNF, brain derived neurotrophic factor; RPLP, 60s ribosomal protein 1.

Briefly, hypothalamic regions were dissected and rapidly snap-frozen in liquid nitrogen. Tissue was homogenized with motor pestle using Trizol reagent (Invitrogen) and removed of decontamination *via* DNase kit protocol (Applied Biosystems). RNA quality was assessed *via* gel electrophoresis, and 1 μ g RNA reverse-transcribed into complementary DNA using the iScript cDNA synthesis kit (Bio-Rad). cDNA product was analyzed by BioRad CFX96 Real Time PCR machine using a two-step PCR and SsoAdvanced SYBR Green Supermix (BioRad) per manufacturer's instructions. Sso7d Fusion DNA polymerase was activated at 95 °C for 30 s. cDNA was then denatured at 95 °C for an additional 30 s following annealing and extension at 55 °C for 40 cycles. After the PCR was complete, specificity of each primer pair was confirmed using melt curve analysis in which each amplicon yielded a single peak. A

cycle threshold ($\Delta\Delta Ct$) analysis was performed with BioRad CFX96 data analysis software and normalized to the reference ribosomal RNA, RPLP.

3. Statistical analysis

Prior to analysis of behavioral data, a D'Agostino–Pearson omnibus test for normality was conducted. If data from behavioral tasks were not normally distributed, a Mann–Whitney U -test was used to account for non-Gaussian distributions. Otherwise, data was analyzed using a student's t -test between conditions. Results were considered statistically significant when $p < 0.05$. Post-mortem GR optical density values and RT-qPCR mRNA values were normalized to respective house-keeping controls (actin and RPLP gene).

4. Results

4.1. Offspring anxiety-related behaviors

4.1.1. Open field

As adults, animals reared and subsequently housed on corncob bedding spent significantly more time exploring the inner arena of the open field relative to wood pulp reared animals ($U(42) = 66.50$, $p < .0001$) (Fig. 1A). More time exploring the inner area of this arena suggests lower levels of anxiety in these animals. Latency to enter the inner arena of the open field as well as number of crosses between quadrants was not significant.

4.1.2. Light–dark box

As adults, animals reared and housed on corncob bedding spent significantly more time exploring the illuminated portion of the light–dark box compared with wood pulp raised animals ($U(32) = 38.00$, $p = .0003$) (Fig. 1B). Greater time exploring the open end of a light–dark box apparatus suggests lower levels of anxiety.

Open field and light–dark box data was not significant for animals placed on respective bedding as adults. Animals which were reared on wood pulp and placed on corn cob in adulthood (open field: $U(26) = 83.00$, $p = 0.7544$ and light–dark box: $U(22) = 45.50$,

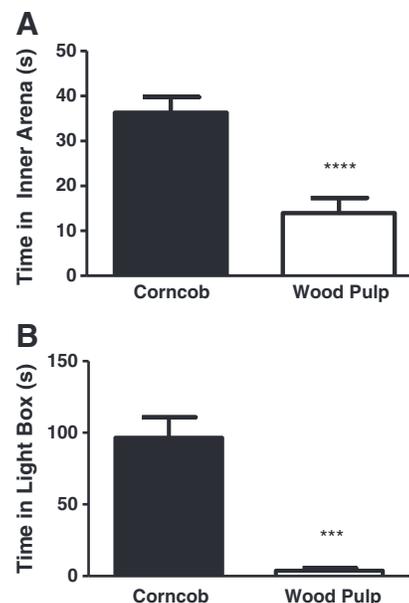


Fig. 1. Offspring behavior in adulthood after rearing on either corncob or wood pulp bedding. A) Open field ($p < .0001$) and B) light dark box ($p < .001$). Results are reported as mean time exploring inner arena of open field and exposed area of light box \pm SEM. Animals raised on wood pulp spend significantly less time on behavioral indices of anxiety as indicated by the open field and light–dark box tasks.

$p = 0.1440$) as well as animals reared on corn cob and placed on wood pulp in adulthood (open field: $t(34) = 1.0$, $p = 0.3170$ and light-dark box: $t(28) = 1.3$, $p = 0.216$) did not differ in behavior (Fig. 2A–D). This suggests that differences in anxiety related behaviors are developmental in nature.

4.2. Glucocorticoid receptor expression and CRH hypothalamic mRNA

As adults, hypothalamic and frontal cortex glucocorticoid receptor expression did not differ between rats housed on corncob or wood pulp bedding conditions ($t(14) = 0.5$, $p = 0.659$ and $t(14) = 0.7$, $p = .4929$ respectively) (Fig. 3A and B). Similarly, CRH peptide, CRH-1R, CRH-2R, and BDNF hypothalamic mRNA were not significantly different between groups ($t(14) = 0.27$, $p = 0.7935$; $t(14) = 0.23$, $p = .8250$; $t(14) = 0.19$, $p = 0.8358$; and $t(14) = 0.24$, $p = 0.8164$ respectively) (Fig. 3C).

4.3. Maternal behaviors

The mean licking and grooming percentages for dams rearing pups on corncob or wood pulp bedding were significantly different. Corncob-housed rat mothers spent significantly more time licking and grooming offspring compared to wood pulp housed dams ($U(10) = 3.5$, $p = 0.0412$) (Fig. 4A). Rat dams across the two groups did not differ in additional measures including i) percent of time arch-back nursing ($U(10) = 14$, $p = 0.8081$) or ii) percent time being on/off nest ($U(10) = 11$, $p = 0.4606$) (Fig. 4B and C).

To control for the putative effects of varying levels of maternal care on adult anxiety measures, animals from both housing conditions were statistically matched for overall levels of maternal care (mean LG score of 7.4%) and performance on anxiety measures assessed. Animals matched for overall maternal care received during the first five postnatal days performed significantly different on both the open field and light-dark box tasks. Rats reared on corncob bedding that had received equivalent amounts of maternal LG as those reared on wood pulp

bedding spent significantly more time exploring the inner area of the open-field ($U(17) = 12$, $p = 0.0079$) (Fig. 5A) and more time in the light portion of the light-dark box ($t(18) = 3.40$, $p = 0.0032$) (Fig. 5B).

5. Discussion

A robust literature highlights the importance of early life environmental variables that influence stress responsivity in adult offspring, primarily using the laboratory rat as a model [1,28,36–39]. One environmental variable demonstrated to influence offspring development is the material on which rodents are housed [40]. In the current study, we employed two different bedding materials commonly used in standard laboratory housing conditions to assess how they may influence anxiety related behaviors in the Long Evans rat. We hypothesized that rearing offspring on qualitatively different bedding materials would influence later measures of adult anxiety-like behaviors and, potentially, underlying neurobiological correlates. Our results demonstrate that varying the bedding on which a laboratory rat is reared contributes to significant differences in anxiety-like behaviors later in adulthood. Rats reared (and subsequently housed) on corncob bedding exhibited significantly less-anxious phenotypes compared to those reared (then housed) on wood pulp bedding material. As glucocorticoid receptor, BDNF, CRH, CRH-R1, and CRH-R2 expression (in various neuronal regions) have all been implicated in the expression of fear and anxiety and are sensitive to early life environmental factors, we wished to assess if bedding conditions during early life influenced the expression of these genes [11,31,41–43]. Hypothalamic CRH, CRH-R1, and CRH-R2 mRNA levels were not significantly different across conditions. Similarly, glucocorticoid receptor expression in the hippocampus and frontal cortex was not significantly different across bedding conditions when measured using western blot. However, this does not preclude the possibility that differences in GR mRNA levels may exist in these regions.

The study of early developmental programming of stress-sensitive phenotypes has most recently focused on the relationship between

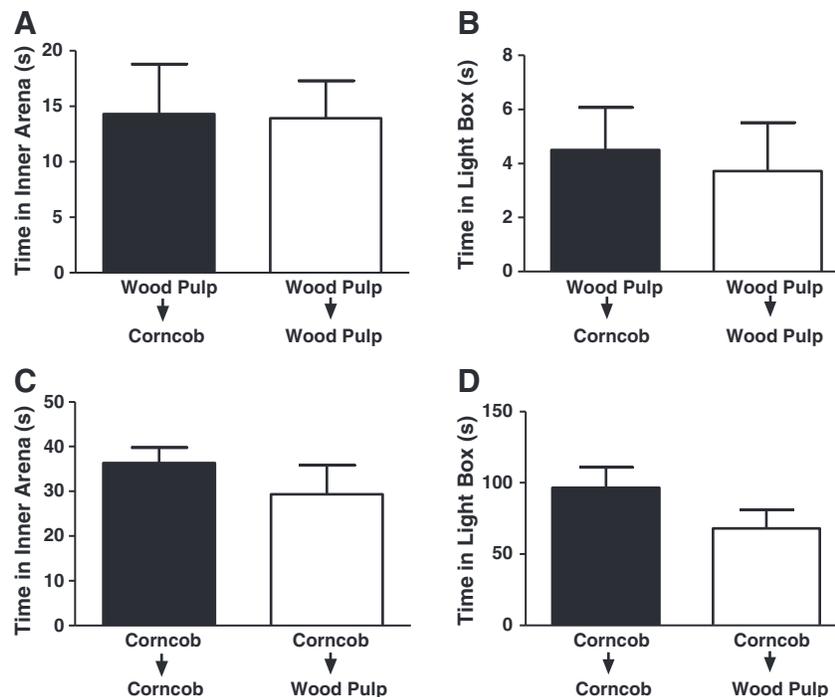


Fig. 2. Offspring behavior reared on corncob or wood pulp bedding and placement on opposite bedding in adulthood. A) Open field ($p > .05$) and B) light dark box ($p > .05$). Animals were reared on wood pulp and moved to corncob bedding in adulthood. C) Open field ($p > .05$) and D) light dark box ($p > .05$). Animals were reared on corncob and moved to wood pulp bedding in adulthood. Results are reported as mean time exploring inner arena of open field and exposed area of light box \pm SEM.

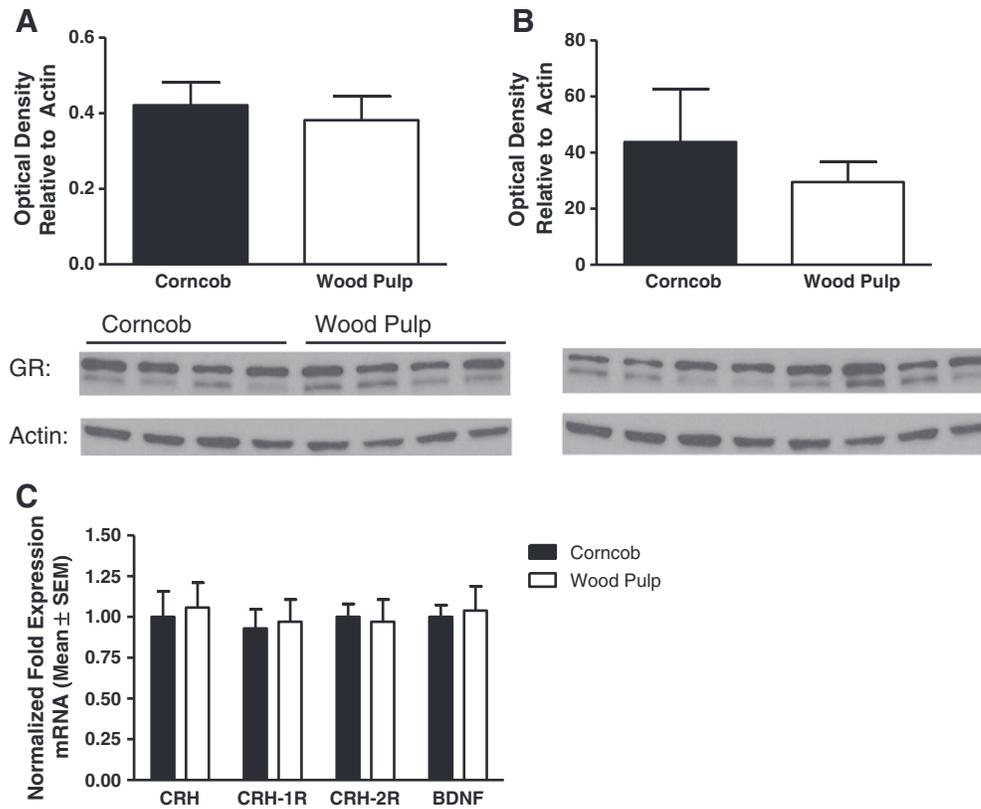


Fig. 3. Post-mortem neural markers. Western blot: A) hippocampal and B) frontal cortex glucocorticoid receptor optical density. Real time-PCR: C) hypothalamic mRNA expression relative to house keeping control gene RPLP. CRH peptide, CRH-1 receptor, CRH-2 receptor, and BDNF mRNA ($p > .05$).

the quality of early life environments and parental care. The extent to which environments directly influence offspring brain and behavior or are mediated by alterations in parental behavior, which subsequently influences offspring development, is subject to much debate (Fig. 6). Evidence supporting direct maternal programming of offspring stress physiology is supported by studies in which an increase in early maternal care has been demonstrated to decrease stress reactivity and anxiety profiles of Long Evans rats later in adulthood [26,28,32]. Ostensibly, our results are consistent with this literature and a ‘maternal mediation’ model, in which maternal investment serves as a link between environments and offspring. We report that offspring reared on corn cob bedding, overall, received higher levels of maternal care as infants, and exhibited lower levels of anxiety-like behaviors as adults relative to offspring reared on wood pulp bedding (mean LG scores of 8.8% and 5.5%, respectively). Interestingly, differences in anxiety related behaviors related to bedding materials are only evident in rats reared under these different conditions. Long Evans rats placed on wood pulp or corn cob bedding as adults do not differ in anxious behavior phenotypes (Fig. 2A–D). This suggests that both maternal care and housing conditions during the early postnatal period are involved in regulating the development of stress-sensitive phenotypes in young offspring. While maternal care provided by rat dams differed across developmental bedding conditions, this factor alone did not fully account for observed differences between groups. To assess direct environmental regulation of stress phenotypes, we matched offspring from both bedding conditions for the quantity of maternal licking and grooming offspring received developmentally. Rats reared/housed on corn cob bedding exhibited significantly less-anxious phenotypes compared to those reared/housed on wood pulp bedding material despite receiving similar levels of maternal care early in life. The bedding material itself, regardless of the maternal care received by the offspring, was sufficient to influence adult stress-sensitive behaviors consistent with direct environmental regulation of adult stress phenotypes (Figs. 5 and 6).

In rodents, previous studies have shown similarly complex associations between early environments, the quantity of maternal care received, and offspring behavior. For instance, in C57BL/6 mice subject to high and variable foraging demand conditions (*i.e.* an unpredictable stressor) maternal care was more active and intense when compared with control mothers in low foraging demand conditions, consistent with a maternal mediation model. However, offspring anxiety-like behavior as adults was varying across gender and condition, with male and female mice responding to environmental cues differentially regardless of overall amounts of maternal care [44]. Similar effects were observed in predation threat paradigms, in which rodent mothers are exposed to predator cues. Exposure to predator odor during the first day of life increases both nursing and licking and grooming provided by rat dams to the offspring during the postpartum period compared with controls. Yet, female offspring of predator odor-exposed mothers display a more anxious behavioral phenotype compared with males, emphasizing the mixed role of environmental regulation of maternal care on offspring behavior [45]. Our results are similar to these findings, showing a nuanced relationship between direct environmental programming and maternal mediation.

Our results clearly emphasize the importance of housing variables in influencing commonly assessed stress-sensitive rodent behaviors, while highlighting the importance of sensitive periods in rodent development. The findings of this paper have important implications for animal husbandry and housing standardization. Behavioral testing across laboratories does not always yield similar results despite rigorous attempts at standardization [46,47]. One unknown variable that may contribute to be disparate behaviors across laboratories may be bedding. Standardization of bedding materials may help reduce inter-experimental variability within and across laboratories by reducing behaviorally anxious phenotypes. Importantly, bedding standardization can also enhance animal welfare by diminishing adult anxiety-like behavior in animals, which is assumed to be deleterious and maladaptive in the laboratory

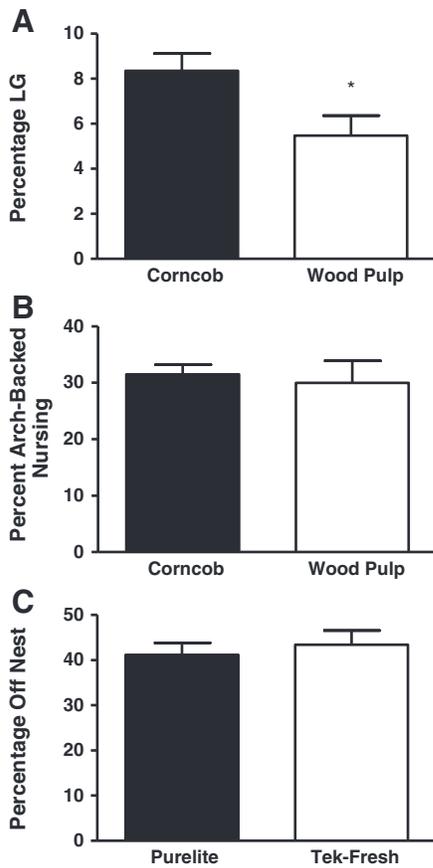


Fig. 4. Maternal behavior observed postpartum. Maternal behavior was observed across the first 5 days post-partum. Behaviors included A) maternal licking ($p < .05$) B) arched-backed nursing and C) time off nest expressed as a percentage of the total number of observations.

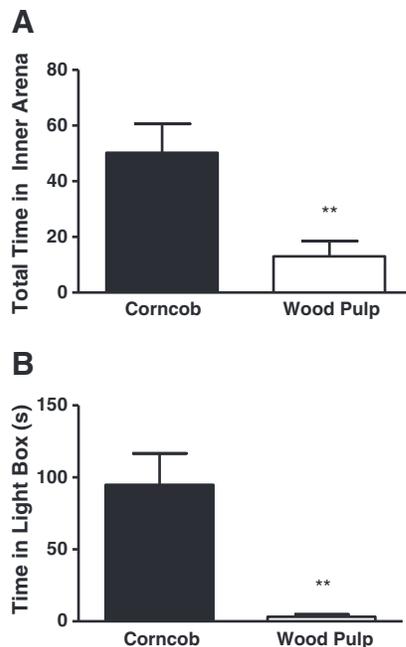


Fig. 5. Behavior of offspring matched for overall levels of maternal care. A) Open field and B) light dark box. Results are reported as mean time exploring inner arena of open field and exposed area of light box \pm SEM.

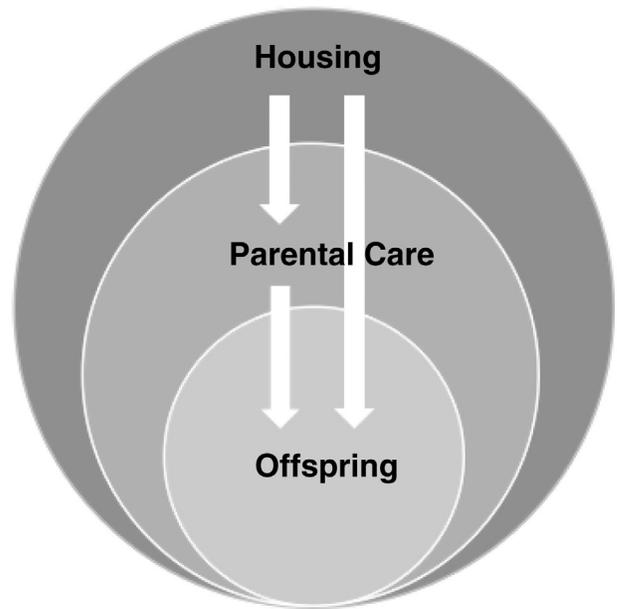


Fig. 6. Schematic representation of housing and parental effects on offspring behavior and physiology.

setting [13,48]. Equally, the conditions in which environments are involved in programming animal behavior may serve as important criteria to refine future research [13]. For instance, when strong anxious phenotypes are required for research purposes, bedding type may also be considered as one avenue to potentiate behavioral effects. For these reasons, we suggest that animal housing parameters, including the type/variety of bedding used in cages, be reported by researchers studying animal physiology and behavior.

The results of this study are in agreement with the few reports examining the effect of bedding materials on adult stress-sensitive measures. Our results are also in line with findings which demonstrate that early-life maternal care received influences offspring behavior later in adulthood. However, some limitations should be considered when interpreting the current results. First, the independent contributions of maternal behavior and bedding type on future offspring behavior require further investigation. We cannot conclude, from the current study, if maternal care and bedding materials are working synergistically or independently to influence the developmental programming of the stress axis. Our data suggest that bedding material is a key component of the developmental programming effect, as animals matched for overall levels of maternal care received early in life still differed in anxiety-like behaviors as adults. We cannot comment more extensively on the role of maternal care in this paradigm as we did not systematically vary the amount of maternal care provided to the offspring; we simply ‘controlled’ for the amount of maternal care received. As mentioned above, while maternal effects on offspring behavior and physiology have been shown extensively in the literature, environmental effects on offspring have also been demonstrated to occur independent of the mother [44].

In the current study we did not investigate the powerful estrogenic properties of corncob bedding. We do not know if early phytoestrogen exposure in rats reared on corncob bedding may be contributing to altered development of the stress-axis and the observed anxiolytic phenotype; however, this is a strong possibility. Phytoestrogens (and the active component, tetrahydrofuranediols) can alter estrogen signaling and estrogen receptor (ER) alpha neuronal expression profiles and may be directly ingested by animals or potentially indirectly absorbed trans-dermally through contact with bedding [23,25,49]. While tetrahydrofuranediols do not bind ER alpha, it has been shown to influence ER levels in the brain and alter behavior by an unknown

mechanism of action [23,49]. Notably, developmental exposure to estrogens (and progestins) has been shown to influence anxiety-like behavior in male and female rodents, acting as an anxiolytic [50–53]. Likewise, through development, intake of phytoestrogens *via* diet has been shown to decrease anxiety-like behavior in Long-Evans rats in a similar direction as shown in this manuscript [54]. The anxiolytic effect of corn cob bedding on rats may be due to perturbations in developmental estrogenic signaling.

In an elegant series of studies using the California mouse, animals housed on corn cob bedding have altered estrogenic profiles and perturbed estrogen dependent behaviors [23]. These effects have also been reported in rats [25]. The most recent paper using the California mouse as a model provides evidence, in line with our own, demonstrating that the effects of corn cob bedding on adult stress-relevant behaviors appears to be generated during the postnatal, developmental window and not in adulthood [55]. An increasing body of research demonstrates that exposure to endocrine-disrupting compounds, particularly during critical developmental windows, may influence sexually-dimorphic neuroendocrine pathways controlling reproductive behaviors. While the bulk of the research using animal models has focused on the role of endocrine disruptors (ED) on various aspects of reproductive physiology, considerably less is known about the role of ED and the stress-axis [56].

As a final point of consideration, given the strong estrogenic properties of both corn cob bedding and laboratory rodent diet, an interactive effect of both food and corn cob bedding may result in adult rats with anxiolytic phenotypes. In the current study, all rats were fed a diet containing 150–250 mg/kg of isoflavones (Tekland Global Diet #2918). Patisaul et al. recently reported that developmental exposure to the ED Bisphenol A (BPA) results in an anxiogenic phenotype in adulthood in rats. However, this anxiogenic phenotype was mitigated if rats were provided with a soy-based diet, demonstrating a strong interaction between EDs in the environment and soy in the diet [57].

In summary, these results suggest that seemingly innocuous environmental variables, such as the choice of bedding material to use in a cage, can drastically alter stress phenotypes of offspring reared on particular types of bedding. It remains to be determined if variables such as phytoestrogen levels or maternal care mediate this effect. We conclude that adult rodent behavior is modifiable by early exposure to differential housing conditions. Lack of attention paid to the potent role this variable plays in the developmental programming of laboratory animals will have deleterious consequences for experimenters and researchers.

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Conflict of interest

All authors declare no conflict of interest.

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