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Environmental Enrichment during Rearing Alters Corticosterone Levels, Thymocyte Numbers, and Aggression in Female BALB/c Mice

Eric K Hutchinson,[1,*] Anne C Avery,[2] and Sue VandeWoude[2]

The goal of environmental enrichment for laboratory animals is to improve welfare, but some enrichment practices may affect research in unintended ways or even be harmful to the animals themselves. We previously found that mice raised at a commercial vendor then given multiple enrichment devices upon arrival at our facilities experienced thymic atrophy and greater variation in measured parameters than did their unenriched counterparts, suggesting that enrichment conditions affected corticosteroid expression in mice. The current study verified and expanded these results, examining 120 female BALB/c mice raised with or without nesting material at a commercial vendor (*n* = 60 per group) and allocated (*n* = 20 per group) to receive no enrichment, nesting material, or 'superenrichment' on arrival at our facilities. Nesting material provided prior to weaning was associated with higher levels of urinary corticosteroid, whereas superenrichment and nesting material during the adult period both led to increased thymic atrophy. Paradoxically, mice that never received enrichment, despite having the lowest corticosterone levels and least thymic atrophy, had increased tail wounds resulting from aggressive interactions. Therefore, enrichment devices that are as seemingly innocuous as nesting material, even if only provided in the preweaning period, may lead to significant, lasting changes in behavioral, physical, or immunologic measures with the potential to alter research outcomes.

The provision of environmental enrichment to mice is practiced widely, yet our knowledge of how different enrichment strategies may affect behavior and physiology has lagged considerably behind implementation. For several decades, most refinement efforts for laboratory rodent husbandry focused on standardizing housing to minimize variability within and between experiments. More recently, as a result of increasing public, regulatory, and internal pressures to improve animal welfare, laboratories have redirected their focus to providing environmental enrichment. The subsequent push to enrich rodent enclosures has resulted in rapid changes in the industry, with surveys demonstrating that most facilities provide some form of structural enrichment.[20] This change in management practice has outpaced the growing body of research examining the positive or negative effects of environmental enhancement schemes. This mismatch is exemplified by the variety of enrichment types used for studies of mouse welfare and the inconsistency in practices at different facilities.[20] In the face of limited reports addressing the influence of environmental conditions on mouse physiology, hypothesis-driven research is needed urgently to ensure that the changes made to rodent environments maximize animal welfare yet minimize potential effects on science.

Environmental enrichment has been defined as "an improvement in the biological functioning of captive animals resulting from modifications to their environment."[32] Studies from several disciplines have demonstrated the positive effects of enrichment on certain measures of welfare in mice. In the field of neuroscience, increased environmental complexity is known to enhance neurocognitive function and plasticity.[6,18,38] Behavior research has demonstrated increased exploration and decreased anxiety as a result of enriched enclosures.[7,33,45,48] Welfare research has confirmed that environmental enrichment may lead to a reduction in stereotypic behavior, as well as fewer wounds and other agonistic interactions, and that mice raised with nesting material require fewer calories to gain and maintain their body weight.[20,33,34,43,52] A consensus has emerged in the literature regarding nesting material in particular as a simple device that encourages normal mouse behavior with minimal effect on commonly measured research parameters.[20,33]

Despite this evidence of positive effects on mouse welfare, some authors have suggested that these and other findings provide evidence that enrichment must be considered a confounding variable.[43] In addition to possible insidious consequences for research, some enrichment devices have in fact been shown to negatively affect certain measures of animal welfare. Superenrichment, the simultaneous provision of 2 or more substantially different devices within a single enclosure, has been associated with negative outcomes including confounding experimental design, higher stress levels in laboratory mice, and negative effects on health.[20,22,33,36,43] In addition, the effects of enrichment on mice have been demonstrated to depend on the strain and sex of the animals, further complicating the search for a single enrichment strategy that can be used effectively for all animals.[1,8,13,26,42,51]

Our previous work demonstrated that BALB/c and CD1 female mice shipped from a commercial vendor and then provided superenrichment as adults displayed immune profiles characteristic of a chronic stress response.[20,47] Enriched mice in this experiment had significant thymic atrophy,

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[1]Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, Maryland; [2]Department of Microbiology, Immunology, and Pathology, Colorado State University College of Veterinary Medicine and Biomedical Sciences, Fort Collins, Colorado.
[*]Corresponding author. Email: ehutchi8@jhmi.edu

antiinflammatory cytokine profiles, and a depletion of CD4 and CD8 double-positive immature thymocytes. These findings, suggestive of a chronic increase in circulating corticosteroids, indicated that superenrichment may in fact be more harmful to mouse welfare than is no enrichment at all and may alter corticosteroid profiles that could affect many types of research studies. We also found that enriched mice had larger standard deviations in nearly every parameter measured, a situation that would necessitate using an increased number of mice per study, because statistical power is decreased as variability surrounding the mean is increased.[20]

In attempting to replicate our previous work, we noted that inhouse-bred BALB/c female mice raised in enriched cages, when compared with BALB/c female mice raised in barren enclosures and shipped directly from a vendor, did not display the differences in thymocyte counts or other physical parameters we had observed between enriched and unenriched animals. This difference led us to hypothesize that the effects of enrichment on adult mice may depend on what enrichment they were provided prior to weaning. We also hypothesized that nesting material is superior to superenrichment in both efficacy and in minimizing effects on common research parameters.

Materials and Methods

Animals. Female BALB/c mice (n = 120) were donated by Charles River Laboratories (Wilmington, MA) and randomly allocated between 2 rearing conditions ('pup condition') at their production facility, with 60 mice raised until weaning in standard, unenriched enclosures (groups with 'None' as the first descriptor; e.g., None-X), and 60 raised with a cotton nesting pad (Ancare, Bellmore, NY; groups with 'Nest' as the first descriptor). In choosing BALB/c female mice, we selected a strain that was reported in 20 of the 41 studies of enrichment identified in a previous review[31] and that we had used for our own previous studies. At weaning (21 d), the mice were shipped to our facility. Each group of 60 mice was allocated randomly into 3 groups of 20 and placed into standard positive-pressure ventilated microisolation cages (4 cages of 5 mice). In addition to autoclaved aspen bedding chips (Harlan Teklad, Madison, WI), the 3 groups were provided one of the following ('adult condition'): no enrichment ('None' as the second descriptor); a compressed cotton nesting pad (Ancare) only ('Nest'); or 3) superenrichment, ('Super,' Figure 1) consisting of a nesting pad, plastic hut (BioServ, Frenchtown, NJ), and 2 plastic balls (BioServ). All cages were changed on a 7-d cycle, with huts, chew toys, and a small portion of nesting material transferred with the mice and a fresh nesting pad added to the new cage, where applicable. Mice were housed on a 12:12-h light:dark cycle and provided pelleted food (8640, Harlan Teklad, Indianapolis, IN) and access to water ad libitum by water bottles. Health surveillance programs performed by the vendor and research institution indicated the mice were free from infections with mouse hepatitis virus, *Mycoplasma pulmonis*, cilia-associated respiratory bacillus, parvovirus, minute virus of mice, pneumonia virus of mice, epizootic diarrhea of infant mice, adenovirus, ectromelia, rotavirus, lymphocytic choriomeningitis virus, cytomegalovirus, polyoma virus, Sendai virus, and *Helicobacter* spp. All research was conducted in compliance with the Animal Welfare Act[3] and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals*.[21] The protocol was approved by the Colorado State University IACUC and was performed in an AAALAC-accredited facility.

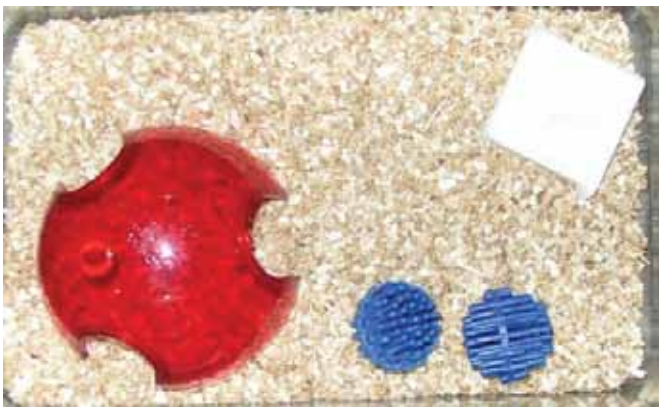

Figure 1. Superenriched cage. Mice in the superenriched condition received a plastic hut, compressed cotton nest pad, and 2 plastic balls; nesting-only mice had only the pictured nesting pad; and unenrichment mice received none of the pictured devices.

Over the 14 wk after their arrival in our facilities, we monitored the mice for effects on general health (body weights, physical examinations), behavior (hair loss, stereotypic behavior, aggression, and fight wounds), hormone excretion (urinary corticosterone:creatinine ratios), and immunologic parameters (thymocyte counts).

Body weight and general health. Immediately on arrival and then every other week, all mice were weighed on a laboratory scale accurate to 0.01 g and inspected for signs of ill health, including wounds and poor body condition.

Hair loss. Immediately on arrival and then every other week, mice were examined and given hair loss scores from 1 to 4 (1, no hair loss; 2, hair loss affecting 1% to 10% of the body surface; 3, hair loss over 10% to 30% of the body surface; and 4, hair loss over more than 30% of the body surface).

Stereotypic behaviors. Beginning the week after arrival and every other week thereafter, each cage was videorecorded for 30 min within 3 to 5 h after the beginning of the dark period. The frequency and duration of all occurrences of stereotypic behaviors were recorded. Stereotypic behavior was defined as bouts of repetitive, apparently aimless behaviors lasting 10 s or more.[53] Such behaviors included but were not limited to: bar gnawing, a bout of repetitive biting into the bars of the cage lid at a particular spot; jumping, rearing at the cage wall followed by either jumping up and down, or upright 'running' with the forelegs against the wall; circling, repeatedly tracing a circle on the cage floor or with forepaws on the cage bars; and bar wheeling, repeated movement from the cage bars to floor.

Aggressive behaviors. The frequency and duration of all occurrences of aggressive behaviors were videorecorded as described earlier. Aggressive behaviors were defined as behavior of an aggressive nature directed at another mouse and eliciting an aggressive or defensive response from that mouse, to include biting, chasing, pinning, mounting, and threat postures.[31]

Tail wounds. Several weeks of homecage observation revealed a pattern of tail-biting aggression. Putatively dominant mice were observed biting or dragging subordinate cagemates away from food or other areas of the enclosure back to the nest (or to a preferred corner of an unenriched cage). Subsequent health examinations, beginning on week 9 and continuing every other subsequent week, included tail-wound scores (1, no wounds; 2, superficial wounds in one focal area of tail; 3, superficial-diffuse wounds or deep-focal wounds; and 4, deep wounds spread diffusely over the tail).

Urine corticosterone:creatinine ratios. Although corticosterone can be measured in serum or feces, we chose to measure urinary corticosterone because this method is less invasive and less subject to handling-associated stress than is obtaining serum[14,44] and, unlike obtaining feces,[9,10,35,50] is not subject to microbial metabolism and can be controlled for differences in output or concentration by comparison to levels of urinary creatinine, a molecule filtered at a constant rate by the kidneys.[13,29,37] On weeks 2, 5, 7, and 12, we noninvasively collected a urine sample from each mouse between the hours of 1000 and 1200. This schedule was maintained consistently to control for circadian variations in baseline corticosterone.[42] Urination was stimulated by transferring the mice individually to clean plastic containers and stimulating their tailbase. Urine was transferred to a sealed microcentrifuge tube by using a 1-mL syringe and stored at −20 °C. On week 12, mice under the adult nesting or unenriched conditions were exposed to an external stressor, a sham intraperitoneal injection, 1 h before urine collection to stimulate corticosteroid release; mice in the adult superenrichment condition were excluded from this time point for technical reasons. After storage, samples were thawed, diluted 50-fold with sterile water and tested for corticosterone by plate ELISA (Corticosterone ELISA kit, Assay Designs, Ann Arbor, MI) and for creatinine by picrate reaction (QuantiChrom Creatinine Assay, Bioassy Systems, Hayward, CA) according to manufacturer instructions. Corticosterone:creatinine ratios were calculated for individual samples and compared as milligrams per mole.

Thymocyte numbers and phenotypes. Corticosteroids have significant effects on the immune system, partially through induction of apoptosis in immature T cells.[4,11,49] This phenomenon can be exploited as an indirect measure of chronic levels of corticosteroids by counting immature T cells in the thymus. In week 14 of the study, mice were euthanized by CO_2 inhalation and whole thymuses collected within 5 min of euthanasia. Thymocytes were harvested by macerating tissue through a biologic screen with a pestle. Samples were washed multiple times in PBS plus 2% FBS. Total cellularity of the sample was estimated by staining with trypan blue and manual counting with a hemocytometer. Cells then were stained with antiCD4–FITC and antiCD8–phycoerythrin surface markers according to manufacturer instructions (BD Biosciences, San Diego, CA) and analyzed by flow cytometry to determine maturity. Cells were gated based on size and scatter properties, with quadrants set by using isotype controls. Dead cells were excluded by using propidium iodide staining. The proportion of immature, double-positive ($CD4^+$ $CD8^+$) thymocytes in comparison to mature, single-positive ($CD4^+$ or $CD8^+$) cells was determined and then multiplied by total cellularity to estimate the total number of immature thymocytes within each mouse.

Statistical analysis. Data are presented as mean ± SEM. Measurements of stereotypic and aggressive behaviors were made by using an entire cage of mice as the subject ($n = 4$ per group). All other measurements were made by using individual mice as subjects. All results were evaluated by using a statistical software package (PRISM, La Jolla, CA, and STATA, College Station, TX). *t* tests were used to compare body weight measurements taken before mice were split into adult groups. Stereotypic behavior was detected only once, rendering further analysis unnecessary. All measurements of hair loss, aggressive behavior, and tail wounds were compiled to reach a cumulative total or average within each subject and then were tested for normality by using D'Agostino–Pearson tests. This process revealed these data to be nonparametric, so groups were subsequently compared by using Kruskal–Wallis analysis, with Dunn posttests where indicated. Thymocyte counts and distributions were analyzed by 2-way ANOVA, using pup condition and adult condition as the 2 factors. Mixed-measures ANOVA (accounting for pup and adult conditions and repeated measures) was used to analyze postarrival weights and urinary corticosterone and creatinine data. Two-way ANOVA allowed us to look for primary effects of pup or adult conditions as well as interaction effects (that is, whether pup condition influenced how mice reacted to adult condition). Mixed-measures ANOVA allowed us to examine data for primary effects of the sampling time point and interactions between time point and housing conditions. Where indicated by ANOVA results, posthoc comparisons between specific groups were made by using the Bonferroni method to correct for multiple comparisons.

Results

Body weight. Weanling mice raised with nesting material arrived significantly ($P < 0.005$) heavier (11.68 ± 0.12 g) than their unenriched counterparts (11.23 ± 0.10 g). Mice raised without enrichment and provided superenrichment at our facility (None–Super group) gained significantly ($P < 0.05$) more weight between arrival and week 3 (6.19 ± 0.18 g) than did mice raised without enrichment and given nesting or no enrichment as adults (None–None, 5.32 ± 0.16 g; None–Nest, 5.29 ± 0.18 g). Three weeks after arrival and at all time points thereafter, differences in body weight between groups were no longer significant.

Hair loss. Very little hair loss was noted throughout the study, and no significant differences were found between groups (Table 1).

Stereotypic behaviors. One instance of stereotypic behavior was observed in a cage of mice that had never received enrichment (None–None group).

Aggressive behaviors. Aggressive behaviors were, for the most part, limited to tail-biting and dragging, but no significant differences were found between groups in the incidence or duration of the behavior itself (Table 1).

Tail wounds. The 6 groups of mice varied significantly ($P < 0.01$) in their tail-wound scores, with mice that never received enrichment (None–None) having the highest wound score (1.68 ± 0.12). This score was significantly ($P < 0.05$) greater than those of mice receiving superenrichment as adults (None–Super, 1.13 ± 0.05; Nest–Super, 1.2 ± 0.09. Tail-wound scores for the remaining 3 groups were 1.40 ± 0.09 for None–Nest, 1.27 ± 0.08 for Nest–None, and 1.33 ± 0.10 for Nest–Nest.

Urinary corticosterone:creatinine ratios. Pup condition had a significant ($P < 0.05$) primary effect on urinary corticosterone:creatinine ratios when analyzed across all time points; mice raised with nesting material had higher levels of urinary corticosterone (99.8 ± 4.0 mg/mol) than did those raised without (83.6 ± 4.0 mg/mol, $P < 0.05$, Figure 2). In addition, hormone levels were affected significantly by which week the sample was taken, with week 7 urinary corticosterone:creatinine ratios (121.4 ± 5.0 mg/mol) significantly higher than those for all other weeks measured (week 2, 84.8 ± 9.0 mg/mol; week 5, 72.4 ± 4.3 mg/mol; and week 12, 78.6 ± 4.7 mg/mol; $P < 0.0001$). Contrary to expectations, corticosterone levels measured after sham intraperitoneal injection were not elevated relative to other time points. The interaction between sampling time point and pup condition was significant ($P < 0.01$), suggesting that the effects of rearing condition varied depending on the week during which the samples were taken. This finding led to subsequent posthoc, week-by-week analysis of pup condition, which revealed that in weeks 5 ($P < 0.01$) and 12 ($P < 0.001$), mice

Table 1. Results (mean ± SEM) from observations of hair loss and aggressive behavior

	None–None	None–Nest	None–Super	Nest–None	Nest–Nest	Nest–Super
Average hair loss score	1.04 ± 0.03	1.08 ± 0.037	1.00 ± 0.00	1.06 ± 0.03	1.00 ± 0.00	1.01 ± 0.01
Total aggressive behavior incidence (no. of observed occurrences)	5.25 ± 4.03	12.00 ± 4.92	5.25 ± 1.03	7.00 ± 3.14	5.00 ± 1.78	3.00 ± 1.00
Total aggressive behavior duration (s)	19.29 ± 13.84	56.05 ± 24.19	27.49 ± 8.89	32.08 ± 14.40	18.82 ± 6.58	10.26 ± 3.78

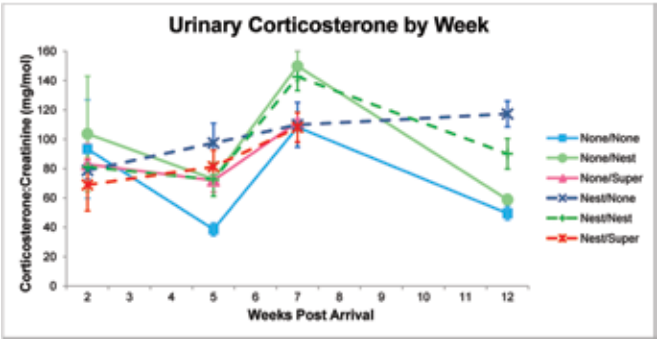

Figure 2. Urinary corticosterone:creatinine ratios by week. Week 12 measurements were made after a sham injection.

raised with nesting material had significantly higher levels of urinary corticosterone (week 5, 83.7 ± 7.0 mg/mol; week 12, 104.4 ± 7.0 mg/mol) than did those raised without (week 5, 61.1 ± 4.7 mg/mol; week 12, 54.1 ± 2.9 mg/mol). The primary effect of adult condition on hormone levels ($P = 0.08$) trended toward but did not reach significance at the 0.05-level. However, the interaction between adult condition and sampling time point was significant ($P < 0.01$), leading to week-by-week analysis of the effects of adult condition on corticosterone. This evaluation revealed that in week 7, mice given nesting material as adults had higher urinary corticosterone:creatinine ratios (146.4 ± 6.8 mg/mol) than did those of other adult conditions (None, 109.1 ± 9.1 mg/mol; Super, 109.9 ± 8.3 mg/mol; $P < 0.01$). The effect of interaction between pup and adult conditions on corticosterone levels ($P = 0.14$) trended toward but did not reach significance at the 0.05-level. At all time points except week 2, the lowest corticosterone:creatinine ratios were found in mice that never received enrichment (None–None group).

In addition to comparing corticosterone to creatinine levels in the urine to correct for differences in urine output or concentration, we compared all groups based on urinary creatinine alone. We found no significant differences between groups, indicating no group-dependent changes in hydration status or urine output (data not shown).

Thymocyte numbers and phenotypes. The adult condition contributed significantly to the differences between groups in total numbers of immature ($CD4^+$ $CD8^+$) thymocytes ($P < 0.05$), with mice provided no enrichment as adults having higher numbers of immature thymocytes ($1.50 \times 10^7 \pm 1.53 \times 10^6$) than those of other adult conditions (Nest, $1.13 \times 10^7 \pm 1.27 \times 10^6$; Super, $1.11 \times 10^7 \pm 1.09 \times 10^6$; $P < 0.01$). However, the interaction between pup and adult conditions was a factor also, indicating that the influence of adult condition on thymocyte numbers depended on previous exposure to enrichment. Posthoc analysis revealed that mice never receiving enrichment (None–None) had the highest total numbers of immature ($CD4^+$ $CD8^+$) thymocytes ($1.84 \times 10^7 \pm 2.11 \times 10^6$; $P < 0.05$), suggesting lower chronic levels of circulating stress hormone, when compared with those of all other groups (None–Nest, $1.07 \times 10^7 \pm 1.48 \times 10^6$; None–Super, $1.00 \times 10^7 \pm 1.27 \times 10^6$; Nest–None, $1.16 \times 10^7 \pm 1.98 \times 10^6$; Nest–Nest, $1.20 \times 10^7 \pm 2.15 \times 10^6$; and Nest–Super, $1.21 \times 10^7 \pm 1.78 \times 10^6$).

Discussion

The current study demonstrates that providing mice with environmental enrichment prior to weaning has both transient and lasting effects on their physiology. In agreement with many previous reports,[20,33] mice that were raised with nesting material as neonates arrived at our facilities weighing significantly more than those raised in barren environments. As has been suggested before, this difference is likely due to the thermoregulatory benefits of nesting material, given that all differences between groups disappeared within 3 wk after arrival (7 wk of age). In contrast to this transient effect, pup-rearing condition exerted a significant direct effect on adult urinary corticosterone levels when analyzed across all sampled weeks, with mice raised in barren cages having consistently lower levels of stress hormone than those of mice raised with nesting material. In addition, the significant interaction between pup and adult conditions with regard to tail wounds and immature thymocyte counts demonstrates that weaning conditions can influence how mice react to their adult environments. These unanticipated and important findings demonstrate the potential for pup-rearing conditions to alter adult physiology in complex ways.

One of the primary objectives of the current research was to determine the possible ramifications of inconsistent housing conditions for preweanling mice, and our results are definitive in this regard. A variable as simple as providing nesting material in the rearing environment may have as much effect on results as experimental variables themselves. Since seminal work with surrogate-reared rhesus macaques in 1958,[17] it has been well documented that conditions during the juvenile period can have lasting and irreversible effects on the behavior and physiology of adult animals. It should come as little surprise, then, that the same holds true for mice, yet only recently in our and other experiments has this possibility been explored with respect to environmental enrichment.[2,15,23,24,27,28,39,40] These findings are of practical importance because many researchers rely on mice delivered from outside sources as the primary subjects of experiments, and our research documents the importance of knowing how mice are being kept prior to delivery.

We also confirmed and expanded our previous results demonstrating that adult mice housed in barren cages have higher numbers of immature thymocytes than do those provided nesting or superenrichment, suggesting lower chronic levels of stress hormone in unenriched mice.[20] In fact, mice never exposed to enrichment materials (None–None) had the highest number of immature thymocytes. These results concur with the urinary corticosterone:creatinine ratios we obtained here in suggesting that a lack of enrichment is associated with lower endogenous production of this hormone. These findings are in contrast to the body of literature suggesting that enrichment, especially nesting material, is beneficial to animal welfare.[20,33]

Existing literature suggests nesting material is superior to superenrichment in terms of benefit to animal welfare and

decreased likelihood of affecting research parameters.[20,33] Providing nesting material or superenrichment to adult mice in our study made no significant difference in any of the measures in which the 2 groups were compared, with the exception of week 7 urinary corticosterone:creatinine ratios, when mice given nesting material as adults actually had higher levels of corticosterone than their superenriched counterparts. We cannot conclude from our data that nesting material as we provided it has any different effect than did super enrichment. A possible explanation for this finding is that the particular nesting material we used for this experiment—compressed cotton nesting squares—may be inadequate for appropriate nest building.[19] Therefore, the effects of nesting material may depend on its character; it is possible that unsuitable types or amounts of nesting material cause distress whereas ample, high-quality nesting materials prevent it.

Although our corticosterone data are strongly supported by the results of our thymocyte measurements, their weekly variations are difficult to interpret and likely resulted from external influences unrelated to the primary experimental design. When compared with putative 'baseline' measurements in other weeks, sham injections at week 12 did not increase corticosterone:creatinine ratios, perhaps indicating either that this procedure was not sufficient to trigger the release of corticosterone or that the timing between the sham injection and urine collection was suboptimal despite our efforts to emulate previously reported methods.[29] In contrast, corticosterone levels measured in week 7 were significantly higher than those measured at all other time points, suggesting that an event prior to sampling that triggered corticosterone release in animals in all groups. Although no such event was identified, any number of activities within a laboratory animal setting could serve as external stressors for mice.

The interpretation of differences in urinary corticosterone and immature thymocyte numbers also is complicated by the lack of published data on these values and the uncertainty of what constitutes physiologically 'normal' exposure to environmental stress. Although our data clearly demonstrate that unenriched mice have lower levels of corticosterone than do mice provided with nesting material or superenrichment, whether these levels are higher than normal in the enriched animals or below normal in the unenriched mice is open to interpretation. Immature thymocytes (that is, those expressing both CD4 and CD8 on the cell surface) are more sensitive than are mature T cells to corticosteroid-induced apoptosis, suggesting our findings represent a true depletion in enriched mice.[49] Although the agreement between our urinary corticosterone and thymocyte results would seem to make corticosteroid-induced apoptosis the most likely explanation, other immunosuppressive conditions somehow affecting only the enriched mice could explain this result as well. Alternatively, the use of a cotton nesting pad in our enriched conditions could have introduced chemical compounds with the ability to act directly on the immune system; a recent study suggested just such a mechanism, showing that cotton balls used for enrichment purposes activated aryl hydrocarbon receptors.[41]

One paradox within the current study is the finding that mice never exposed to enrichment (None–None) had the most severe tail wound scores despite lower direct and indirect measurements of corticosterone. From a behavioral and animal wellbeing standpoint, tail wounds and the aggressive interactions would likely be at least equally important as other measured physiologic changes. However, our behavioral measures did not reveal any significant differences among groups, perhaps because of low incidence and small group size. Had we used more mice in the current study or designed it to collect aggression and stereotypy data from more than 4 cages per group, we may have been able to further explore this contradiction between our physiologic and behavioral measures.

Our results raise many additional questions about the basis for differences between mice raised with or without nesting material. Perhaps such differences could be related to the well-documented effects of nesting material on neonatal thermoregulation and subsequent caloric intake and weight gain. A plausible possibility is that neonates struggling to maintain energy balance and body heat could suffer dysfunction in other developing organ systems as a result. In addition, although nest building in mice is an instinctual rather than learned behavior, nest quality is known to vary between strains[5] and potentially could depend on puphood experiences; the ability to build a better nest as an adult could lead to differences in microenvironment, which in turn could translate to changes in stress or other physical parameters. Furthermore, the presence of nesting material could lead to differences in maternal physiology, subsequently leading to lasting differences between pups. Maternal stress has been shown to produce lasting alterations to the hypothalamic–pituitary–adrenal axis in rodent pups,[8,30] an effect that could help explain the differences in corticosterone and thymocyte numbers observed in our experiment. Finally, the consequences of enrichment on neural development, long studied by the field of neuroscience, could contribute in any number of ways to our findings and largely remain unexplored in terms of effect on animal welfare.[12,16,25,39,46]

The effects of environmental enrichment have repeatedly been shown to be affected by sex and background strain.[1,8,13,26,42,51] Although we chose to use BALB/c female mice owing to the strain's frequent use in enrichment and other research, including our own, our behavioral measures of stereotypy, aggression, and hair loss would no doubt have been different had we chosen to study C57BL/6 male mice instead. Further studies are necessary to determine whether the findings presented here could be duplicated in male or other strains of mice and whether alternative means of assessing welfare would support the conclusion that enrichment leads to higher levels of stress.

Our findings emphasize the point that environmental enrichment strategies in common use today may not benefit all mice as intended. Regardless of whether a relative decrease in corticosterone actually reflects improved welfare, environmental enrichment must be considered a variable like any other, with real ramifications for physiology, including measurable effects on the immune system. In light of both of these conclusions, investigators, regulatory agencies, and laboratory animal professionals should insist upon evidence-based environmental enrichment strategies that are of demonstrable benefit to the animals or, at the very least, strategies whose potential effects on experimental outcomes are well-considered.

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