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Cage Change Influences Serum Corticosterone and Anxiety-Like Behaviors in the Mouse

Skye Rasmussen,[1,3,*] Melinda M Miller,[2] Sarah B Filipski,[2] and Ravi J Tolwani[1]

Environmental variables and husbandry practices can influence physiology and alter behavior in mice. Our study evaluated the effects of cage change on serum corticosterone levels and anxiety-like behaviors in C57BL/6 male mice. We examined the effects of 3 different methods of performing cage transfer and of transferring mice to a clean or a dirty familiar cage microenvironment. The 3 different handling methods were forceps transfer, gentle transfer with gloved hands, and a passive transfer technique that did not involve active handling. Active handling methods and transfer to both clean and dirty cage microenvironments significantly increased serum corticosterone 15 min after cage change; however, at 60 min after cage change, levels were comparable to those of unmanipulated mice. Although the effects were transient, cage change altered anxiety-like behaviors in the open field when behavioral testing was performed on the same day. These results demonstrate that the timing of cage change can influence behavioral results, an effect that is an important consideration for rodent behavioral studies.

General husbandry practices can have myriad effects on animal physiology and behavior. As the number of studies that incorporate behavioral testing into their molecular and genetic profiling increases, the epigenetic effects of the housing environment are becoming increasingly evident. Seemingly negligible variables, such as enrichment, can have profound effects on brain structure, function, and neurophysiology.[14,17-19,23] Alterations of the light cycle, noise levels, rack position of the home cage, and home cage disruption have been shown to affect breeding, physiologic or behavioral measures of anxiety, and occasionally experimental outcome.[1,4,10,13,20,21,24,25] Husbandry practices that induce physiologic changes in stress hormones can have both positive and negative implications for behavioral testing. Previous behavioral studies have demonstrated that elevated corticosterone levels can either impair or improve cognitive performance, depending on the individual circumstance.[8,22,30]

Historically cage change has been used to create a model of hypertensive stress in mice,[15,16,29] and multiple studies have demonstrated a correlation between cage change and physiologic stress responses in rodents.[1,4,5,7,21,26] The components of cage change that contribute to rodent stress and anxiety are multifaceted. Cage change most often takes place during the light phase of the light:dark cycle, at a time when mice are less active or resting. Animal husbandry staff manipulate or handle the mice during the cage transfer process. After cage transfer, the new clean cage microenvironment no longer contains the urine scent markers or pheromones mice use for social identification and hierarchical perception. Male mice respond with increased activity levels and fighting,[27,28] and breeding mice have been reported to have increased pup mortality[21] and a higher incidence of cannibalism[4] after cage change.

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[1]The Comparative Bioscience Center and [2]Harold and Margaret Milliken Hatch Laboratory of Neuroendocrinology, The Rockefeller University; and [3]The Tri-Institutional Training Program for Laboratory Animal Medicine and Science, Memorial Sloan-Kettering Cancer Center, The Rockefeller University, and Weill Cornell Medical College, New York, New York.
[*]Corresponding author. Email: srasmussen@rockefeller.edu

Whether handling or providing a clean cage microenvironment is of equal or greater significance to mice is debatable. Handling consistently generates a corticosterone response in rodents,[1] and mice, unlike rats, habituate poorly to handling.[1,9,12] In contrast, aggression between male mice after cage change is reduced if nesting material is transferred from the dirty cage,[27] suggesting that the loss of scent markers is an important factor.

We designed a 2-part study to further examine the effects of cage change on the physiology and behavior of C57BL/6 male mice. Our study assessed 3 different cage-change handling techniques: handling with forceps, gentle handling with gloved hands, and a passive transfer technique. Forceps transfer was performed in the same efficient manner used by the animal care staff. Gloved transfer consisted of a gentler and deliberately slower transfer technique than was forceps transfer. A passive transfer technique was devised to eliminate direct handling and consisted of using a pair of forceps to gently herd mice between 2 tilted cages. Depending on their experimental group, mice were either transferred to a clean cage, with no residual pheromones or urine scent markers, or mice were returned to their original dirty home cage.

Part one of our study evaluated serum corticosterone levels at 15 and 60 min after cage change. We hypothesized that serum corticosterone levels would be higher for experimental groups of mice subject to forceps transfer than they would be for groups of mice manipulated with slower or gentler handling techniques. We also hypothesized that mice would have higher serum corticosterone levels if they were transferred to a clean cage microenvironment. The second part of our study used 2 behavioral tests—the open-field test and elevated-plus maze—to assess anxiety-like effects of cage change on behavior. Our hypothesis was that mice would be more likely to exhibit behaviors consistent with anxiety after cage change. We predicted that anxiety-like behaviors would be most evident in groups of mice that underwent forceps transfer or were transferred to clean cages. The results of our study revealed transient elevations in serum corticosterone in all groups of mice, and anxiety-like behaviors were altered when mice were assessed

behaviorally on the same day of cage change, regardless of handling method.

Materials and Methods

Mice. All research was IACUC-approved and was conducted at an AAALAC-accredited facility. Male C57BL/6J mice (n = 221; age, 6 wk; Jackson Labs, Bar Harbor, ME) were assigned to experimental groups for the purpose of either testing anxiety-like behavior or evaluating serum corticosterone levels. Mice that were designated for use in the behavioral studies were not used for the serum corticosterone assays. Mice were housed 4 per cage in standardized ventilated microisolation caging (Thoren, Hazelton, PA) measuring 67 in.^{2}. Mice had free access to irradiated feed (LabDiet 5053, Purina Mills International, St Louis, MO) and bottled chlorinated (2 ppm) water. Each cage contained 400 mL irradiated corncob bedding (Bed-o-cobs, The Andersons, Maumee, OH) and was supplemented with a synthetic nesting material (Enviro-dri, Shepherd Specialty Papers, Milford, NJ). Bedding was changed once weekly by the investigator within a ventilated cage-change station. All mice were housed in the same rodent housing room. The housing room was maintained on a 12:12-h light:dark cycle (lights on, 0500 to 1700), and room temperature ranged between 20 and 22.2 °C (68 and 72 °F). The relative humidity ranged between 30% and 70%. Room air changes were set at 10.5 changes hourly, with the ventilated racks providing 50 air changes hourly at cage level. Dirty-bedding sentinels were screened quarterly by using serology and parasitology and were found to be negative for commonly encountered murine pathogens.

Serum corticosterone level analysis. ***Experimental group 1.*** To evaluate the effects of handling compared with the effect of exposure to clean or dirty bedding, mice were housed 4 to a cage. Cage change by forceps transfer was performed once weekly by the primary investigator for a period of 2 wk. Seven days after the last cage change, mice were assigned to 2 experimental groups, a dirty bedding group (48 mice) and a clean bedding group (48 mice). Each group was experimentally manipulated using 1 of 3 handling techniques: forceps transfer (16 mice), gloved transfer (16 mice), and passive transfer (16 mice; Figure 1 A). Mice were moved to either the original, familiar dirty home cage or a new clean cage. During forceps transfer, mice were caught anywhere along the body by using the forceps and briefly lifted. During gloved transfer, the investigator used one hand to grasp each mouse firmly by the tail with one hand and supported under the legs with the other hand as the mouse was lifted. The passive transfer technique consisted of gently herding mice with a pair of forceps between 2 cages tilted together at a 45°-angle. Each handling method was timed; forceps transfer took an average of 8 s to complete, gloved transfer averaged 14 s, and the passive transfer technique took the longest, averaging 24 s to complete. All experiments were performed between 1500 and 1700 h. At the time of euthanasia, the primary investigator performed the 3 different cage-change handling methods for each experimental subgroup. Eight mice from each experimental subgroup were euthanized by cervical dislocation at 15 and 60 min after cage change; terminal blood samples were collected for corticosterone level analysis.

Experimental group 2. To further evaluate the effects of the individual handling techniques, mice were housed 3 to a cage for 3 wk. Because mice potentially are acclimated to forceps transfer at the vendor facility, an acclimation period was provided for the novel handling techniques. Experimental groups were divided by handling method: forceps transfer (12 mice), gloved transfer (12 mice), and passive transfer (12 mice). For the duration of the acclimation period, the primary investigator performed cage changes weekly by using the techniques designated for each experimental group. Cage change was performed between 1500 and 1700 h. On week 3, the primary investigator performed the final series of cage changes for the 3 experimental groups. All mice were transferred to clean cages with clean bedding. After cage change, 6 mice from each group were euthanized at 15 and 60 min after cage change. Mice were euthanized by cervical dislocation, and terminal blood samples were collected to assess corticosterone levels.

Serum corticosterone control group. Five mice were euthanized by cervical dislocation between 1500 h and 1700 h to assess basal corticosterone levels. Mice were euthanized 7 d after the last cage change. Two serum samples were inadequate in volume and therefore were eliminated from analysis.

Serum corticosterone assays. Terminal blood samples were centrifuged and serum separated and frozen (below −15 °C) prior to testing. A commercial serum corticosterone radioimmunoassay kit (Rat/Mouse Corticosterone RIA 1251, MP Biomedical Solon, OH) was used. Serum samples were randomized and assayed in duplicate. The assay had high and low limits of detectability of 5 and 1000 ng/mL, respectively, according to a standard curve. All procedures were performed according to the manufacturer's instructions. Final values were determined by averaging the results of duplicated samples.

Behavioral analysis. ***Experimental group 1.*** To evaluate the behavioral effects of transferring mice to the original dirty home cage or to a clean cage devoid of scent markers, mice were housed 4 to a cage. After arrival at our facility, cage change occurred once weekly for a period of 2 wk and was performed by a designated member of the husbandry staff using the forceps transfer technique. On the third week, mice were assigned to 3 cage-change experimental groups: a dirty bedding group (12 mice), a clean bedding group (12 mice), and a control group (12 mice; Figure 1 B). Cage change occurred 7 d prior to behavioral testing in the control group. On day 1 of behavioral testing, the forceps transfer technique was performed by the designated husbandry staff member at 1500 h.

Experimental group 2. To evaluate potential behavioral effects linked to the 3 cage-change handling methods, mice were housed 4 to a cage. Mice were assigned to 4 cage-change experimental groups divided by handling method: forceps transfer (12 mice), gloved transfer (12 mice), passive transfer (12 mice), and a control group (12 mice; Figure 1 B). Cage change occurred 7 d prior to behavioral testing in the control group. The primary investigator performed cage change by using the experimentally assigned handling methods once weekly for 3 wk. On the fourth week, cage change was performed at 1500 h by the primary investigator using the experimentally assigned handling methods, and day 1 of behavioral testing was initiated.

All behavioral studies were performed in a designated procedure room located within the vivarium. Transport to the room occurred no sooner than 30 min after the end of the light phase of the light:dark cycle (1750 h). Mice were acclimated to the procedure room for 1 h. Behavioral testing took place between 1850 and 2250 h and occurred in the following sequence: day 1, open-field test; day 2, elevated plus maze (Figure 1 B). The sequence of behavioral testing was selected based on previous recommendations that encourage increasing the novelty of tests over the course of the testing battery to help to avoid decreased exploratory behavior due to boredom.[6] Animal activity was observed and recorded for 5 min by using behavioral tracking software (Ethovision XT, Noldus Information Technology, Leesburg, VA), and equipment was cleaned with 50% ethanol

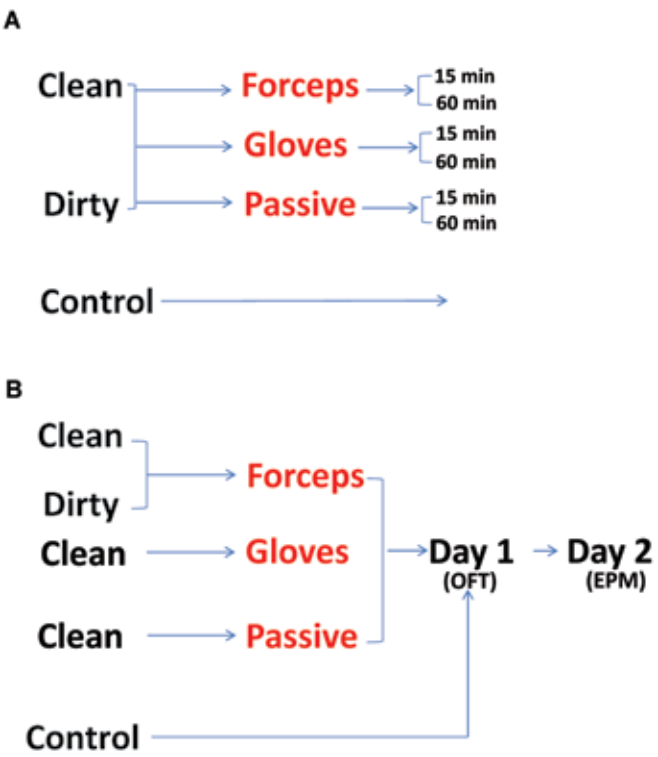

Figure 1. Illustration of experimental design for (A) serum corticosterone level analysis and (B) anxiety-like behavioral assays. OFT, open field test; EPM, elevated plus maze.

after individual trials. Mice were tested individually in a randomized pattern that was repeated in the same sequence over the 2 consecutive days of behavioral testing.

Open-field test. The open field was custom-constructed by using acrylic (45 × 45 × 20 cm). The center zone measured 19.7 cm^2. Tests were conducted under low-light conditions (25 lx). At the start of each behavioral trial, mice were placed in the same corner zone. The center point of the mouse was videotracked to record average velocity, total distance traveled, time spent in the center zone, and time spent around the perimeter of the open field.

Elevated plus maze. The elevated plus maze was constructed of acrylic. Both open and closed arms individually measured 30 cm in length. All experiments were performed under low-light conditions (25 lx) as measured by a lux meter positioned at the periphery of open arms. At the beginning of each trial, mice were placed in the center of maze facing a closed arm. Videotracking was used to record the time spent in the open and closed arms and numbers of entries into open and closed arms over the duration of the trial. An entry was scored when the center point of the mouse entered an arm.

Statistical analysis. Statistical analysis was performed by using a standard statistical software program (GraphPad Prism 5, GraphPad Software, La Jolla, CA). One-way ANOVA and Dunnett posthoc tests were used to compare individual experimental groups. Some analyses required the use of 2-way ANOVA. Either Tukey or Bonferonni posthoc tests were selected to adjust for multiple comparisons among experimental variables.

For corticosterone assays, the mean value of the duplicated corticosterone samples was analyzed. Total distance traveled, time spent in the center zone, and time spent in the perimeter zone were used for statistical analysis of the open-field test. Statistical analysis of the elevated plus maze included analysis of the time spent in and numbers of entries into both the open and closed arms.

Results

Corticosterone assays. No statistical differences in serum corticosterone levels were found when comparing cage-change handling methods in groups of mice that were not acclimated to the various handling methods; therefore, experimental groups were combined to analyze the effects of transfer into a clean or dirty cage. Whereas the corticosterone levels between the clean and dirty bedding transfer groups did not differ, corticosterone levels at 15 min after cage change in both cage-change groups were significantly ($P = 0.0208$) different from that of the unmanipulated control group (Figure 2 A). This effect was transient: corticosterone levels of mice euthanized at the 60-min time point were not different from that of the unmanipulated control group ($P > 0.05$).

Corticosterone levels in mice that were acclimated to the experimental cage-change techniques for 3 wk differed significantly ($P = 0.0362$) between groups at the 15-min time point (Figure 2 B). Post hoc analysis revealed that corticosterone levels were higher in mice that were handled with forceps or gloves than in the control group. Serum corticosterone levels of the passive transfer group did not differ significantly from those of the control mice. At the 60-min time point, serum corticosterone levels were not significantly different between the 3 handling techniques and the control group ($P > 0.05$).

Behavioral tests. The open-field test was performed on the same day as cage change. The duration of time each mouse spent in the peripheral and central zones was analyzed. Clean and dirty bedding groups were compared by using one-way ANOVA. No statistical difference was noted in anxiety-like behaviors exhibited by clean or dirty bedding groups ($P > 0.05$). A one-way ANOVA of the cage change groups revealed a trend toward a difference in anxiety-like behaviors between groups (Figure 3 A, $P = 0.10$). No significant behavioral differences were detected between the individual handling methods ($P > 0.10$), indicating that any manner of previous handling or manipulation may possibly alter behavior. When cage-change groups were collapsed across method, there was a significant ($P = 0.0011$) difference between cage change and control groups (Figure 3 B). Experimental groups undergoing cage change earlier that day spent more time in the center zone of the open field when compared with the control group. Total distance traveled did not differ statistically between groups ($P > 0.10$).

Analysis of the elevated plus maze on the second day of behavioral testing demonstrated no significant difference between experimental groups as compared with the control group (Figure 3 C, $P > 0.39$).

Discussion

The current study evaluated the physiologic and behavioral effects of cage change on C57BL/6J male mice. We compared the effects of various handling methods used during cage change as well as the effects of alterations in the cage microenvironment. In addition, we sought to identify the duration of those effects on anxiety-like behaviors.

Mice transferred to clean and dirty cage microenvironments both demonstrated a transient increase in serum corticosterone levels. However, serum corticosterone levels did not differ statistically between clean and dirty bedding transfer groups. Therefore, the different olfactory and hormonal factors did not detectably affect corticosterone response, These findings

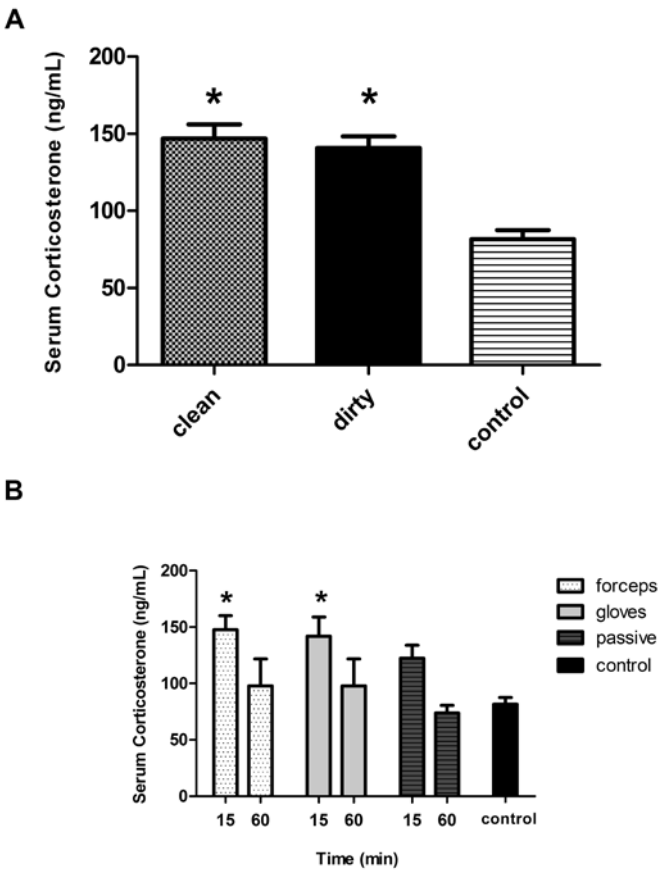

Figure 2. (A) Serum corticosterone levels (mean ± SEM) of clean and dirty bedding transfer groups at 15 min subsequent to cage change. (B) Comparison of serum corticosterone levels (mean ± SEM) associated with individual handling methods at 15 min and 60 min subsequent to cage change. *, Values for forceps and gloves transfer methods significantly ($P < 0.05$) different at 15 min compared with control value.

were contrary to our original hypothesis, which predicted that transferring mice to a clean cage devoid of scent markers would result in higher serum corticosterone levels.

In contrast to transfer to a clean or dirty cage, the transfer process itself increased corticosterone levels. Transfer methods that involved active handling of mice, whether with forceps or gloved hands, induced significant increases in serum corticosterone levels at 15 min after cage transfer. In contrast, the serum corticosterone levels of the minimally handled, passive transfer group did not differ statistically from that of the unmanipulated control group. Furthermore, serum corticosterone levels were lower in the passive transfer group even though this technique required a longer period of mouse manipulation for completion of the transfer. To our knowledge, this study is the first to demonstrate that active handling of mice during cage transfer increases serum corticosterone levels to a greater extent than does passive transfer.

Typical of an acute stressor, serum corticosterone level elevations associated with active handling cage transfer techniques returned within 60 min to levels similar to those of unmanipulated control mice. This transient surge in corticosterone subsequent to handling is consistent with other studies.[2,9]

Although the physiology of the mouse may normalize shortly after cage change, alterations in behavior may persist. Differences in anxiety-like behaviors were detected in the open-field test when it occurred on the same day as cage change. These

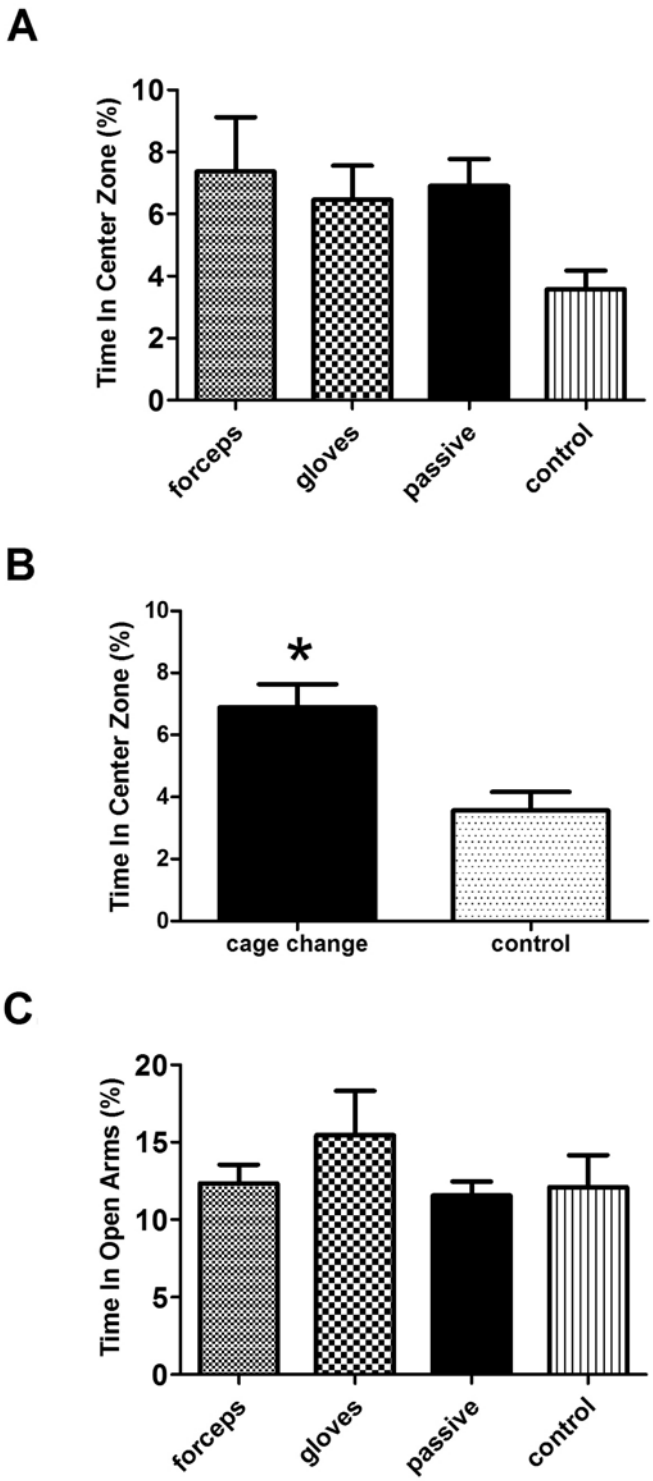

Figure 3. (A) Percentage (mean ± SEM) time spent in the center zone of the open-field test on day 1 of behavioral testing. Handling groups demonstrated a trend toward greater time spent in the center zone ($P = 0.10$) when compared with the control group in a one-way ANOVA. (B) Percentage time (mean ± SEM) spent in the center zone of the open field comparing combined cage change groups to the unmanipulated control group. *, Value for cage change group significantly ($P < 0.05$) different from that of control group. (C) Percentage time (mean ± SEM) spent in the open arms of the elevated plus maze on day 2 of behavioral testing.

effects were present in all experimental groups of mice handled or manipulated for cage change, regardless of whether the cage-change method significantly altered serum corticosterone

levels. Although corticosterone levels increased as a result of cage change, the levels were not as high as those found in acute stress experiments with more severe stressors.[3] Previous studies examining the relationship between corticosterone levels and behavior vary widely in reported effects on anxiety and locomotion.[11,22,25] Our finding that a small, transient increase in corticosterone after cage change corresponded with increased time spent in the center of the open field is consistent with previously reported variations in rodent behavior.[1] The current study indicates that various forms of handling or environmental disruption can lead to altered behavior, and this effect may confound or modulate behavior being examined during an experiment. However, the behavioral effects of cage change are not long-lived, as shown by testing for anxiety-like behavior at 2 d after cage change. Behavioral testing in the elevated plus maze conducted 24 h after cage change indicated no residual behavioral effects.

In conclusion, we determined that physiologic indicators of stress in rodents, such as serum corticosterone levels at the time of behavioral testing, do not always predict the effect of an environmental stimulus on anxiety-like behavior. Standard husbandry practices, such as cage change, can alter anxiety-like behaviors in mice tested many hours later. Investigators studying rodent behavior should consider this issue when planning behavioral studies. To minimize experimental variables, investigators should be aware of cage change schedules for their mice and plan experiments accordingly.

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