Journal of the American Association for Laboratory Animal Science
Copyright 2022
by the American Association for Laboratory Animal Science

Vol 61, No 1
January 2022
Pages 42–51

Behavioral and Physiologic Effects of Dirty Bedding Exposure in Female ICR Mice

Anne L Merley[1], Jennifer S Hubbard[1], Aaron K Rendahl[2], Felicia D Duke Boynton[3], Lynn Collura Impelluso[1,2,*]

Exposure of sentinel mice to dirty bedding is commonly used in health monitoring programs to screen colonies for clinical and subclinical disease. Despite the potential stressors present in dirty bedding, including but not limited to microorganisms, pheromones, and ammonia, it is unknown whether sentinel mice exposed to soiled bedding experience stress. In this study, select behavioral and physiologic changes associated with stress were assessed in female ICR mice exposed to dirty bedding. Behavioral parameters included evaluation in the home cage and selected behavioral tests; physiologic measurements included neutrophil:lymphocyte ratio and weight. Mice in the acute group were exposed for 24 h whereas mice in the chronic group were exposed for 4 wk. Mice in the chronic group exposed to dirty bedding weighed less at days 21 and 28 than did control mice. Chronic mice exposed to dirty bedding also exhibited decreased net weight gain over the entire study period as compared with control mice. No significant differences were detected in the other behavioral and physiologic parameters measured. These results indicate that dirty bedding exposure may affect sentinel mice, but further investigation is needed to determine the specific mechanism(s) behind the weight difference.

Abbreviations: ICC, intraclass correlation; NLR, neutrophil:lymphocyte ratio

DOI: 10.30802/AALAS-JAALAS-21-000060

Introduction

Regular health surveillance is a critical component of colony management for laboratory mice. Microbial infections have the potential to cause clinical and subclinical disease, resulting in detrimental effects on animal welfare and could lead to experimental variability. Although the goal of identifying adventitious microorganisms in colony animals is the same across institutions, the design of the health surveillance program can vary greatly and is tailored to the individual facility. Health surveillance methods include direct sampling of either the resident population or indirectly exposed sentinel mice and environmental monitoring. Despite these options, many programs rely on the use of sentinel mice exposed to dirty bedding. These mice are dedicated to use for regular exposure to pooled dirty bedding from colony animals.[51] Relying on fecal-oral transmission, these sentinel mice are assumed to reflect the microbiologic status of the resident population and are regularly tested to help identify infections in the larger colony. This health surveillance system prevents disruption of the resident research population and allows one or a few sentinel mice to monitor multiple cages at once, decreasing the cost and burden of testing.[49]

Despite the advantages and widespread acceptance and use of sentinels exposed to dirty bedding, the effects of exposure to a potentially aversive material on the welfare of the sentinel mice may be a concern. In addition to microorganisms, dirty bedding contains other stimuli (for example pheromones, ammonia) that can act as sources of stress.[3] Pheromones are species-specific chemical signals that are secreted into the environment by an individual and affect the behavior and physiology of conspecifics.[11] Pheromones mediate reproduction and receptivity,[10,34,36,72,87,88,92] aggression and dominance,[14,50,65,74] and defensive behaviors.[6,9,76] These volatile chemicals are detected by the sensory neurons in the main olfactory epithelium and/or vomeronasal organ, which relay the signal to the central nervous system eliciting a response.[57] The presence of pheromones in bodily secretions, including but not limited to urine and exocrine glands, allows them to be transferred efficiently in dirty bedding.[41,43,58,59,95] These chemical signs, especially defensive or alarm pheromones, can elicit a stress response.[38,42] In addition to pheromones, dirty bedding may be a source of ammonia. Ammonia is produced in rodent cages by the conversion of urea present in urine to ammonia by bacterial urease enzymes. Levels of ammonia in the cage gradually increase after cage change, although the magnitude of this increase depends on the housing and environmental conditions.[13,22,29,37,48,55,68,69,75,82,91] Ammonia is an irritant that can affect the upper airways, and exposure to elevated levels has been associated with pathologic lesions in rodents.[12,13,29,55,91] The effects of these unintended components of dirty bedding on sentinel mice are currently unknown.

Previous studies have used dirty bedding as a source of olfactory-mediated psychosocial stress in mice.[46,47] In this test, known as cage switch or exchange, individual male mice are moved to a cage previously occupied by an unrelated male mouse.[46,47] The mice exposed to the dirty bedding exhibit characteristic acute changes consistent with stress, including hypertension,[46,47] increased locomotion,[44,46] and increased serum adrenocorticotropic hormone.[8] Although the conditions of the cage switch stress test appear to mimic the experience of dirty-bedding sentinel mice, several differences prevent a direct comparison, including the sex of the mice, source of the bedding, and length of exposure; dirty-bedding sentinel mice are usually female and are exposed to bedding from multiple cages of mice over long periods of time.[49] Despite or perhaps because of these differences, sentinel mice may experience stress

Received: 26 May 2021. Revision requested: 15 Jul 2021. Accepted: 23 Sep 2021.
[1]Research Animal Resources, University of Minnesota, Minneapolis, Minnesota, [2]College of Veterinary Medicine, University of Minnesota, Saint Paul, Minnesota, [3]Department of Comparative Medicine, Mayo Clinic, Rochester, Minnesota
[*]Corresponding author. Email: impell@umn.edu

as a result of exposure to dirty bedding. Therefore, the objective of this current study was to evaluate the behavioral and physiologic effects of exposure to dirty bedding on sentinel mice.

Female ICR mice exposed to pooled dirty bedding from breeding and experimental colony mice were compared with control mice exposed only to clean bedding. Behavioral parameters measured included home cage evaluation of hair coat and behavior (nest building, stereotypical behavior) and behavioral tasks (light-dark box, elevated plus maze). Neutrophil:lymphocyte ratio (NLR) and weight were evaluated as physiologic measurements of stress. The estrous cycle stage was determined at the end of the experiment and analyzed as a covariate. The effects of exposure to dirty bedding were evaluated at 2 time points: 24 h (acute) and 4 wk (chronic). The hypothesis was that exposure to dirty bedding would result in behavioral and physiologic changes consistent with stress at both time points.

Materials and Methods

Animals. Female Crl:CD1(ICR) mice (n = 80, 23- to 25-d-old and 13 to 15 g at arrival) were obtained from Charles River Laboratories (Wilmington, MA). Based on vendor assessment, these mice were free of the following pathogens and opportunistic organisms: Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvovirus, murine norovirus, Theiler murine encephalomyelitis virus, reovirus, murine rotavirus, lymphocytic choriomeningitis virus, ectromelia virus, mouse adenovirus, mouse cytomegalovirus, K virus, polyoma virus, Hantaan virus, mouse thymic virus, lactate dehydrogenase-elevating virus, *Bordetella bronchiseptica*, *Citrobacter rodentium*, cilia-associated respiratory bacillus, *Corynebacterium kutscheri*, *Helicobacter* spp., *Klebsiella* spp., *Mycoplasma pulmonis*, *Pasteurella* spp., *Salmonella* spp., *Staphylococcus aureus*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, β-hemolytic *Streptococcus* spp., *Clostridium piliforme*, *Encephalitozoon cuniculi*, ectoparasites, and endoparasites. Upon arrival, the mice were housed in pairs in standard polycarbonate static microisolation rodent cages (Allentown 75 Static with low-profile lid; Allentown, Allentown, NJ). The mice were housed on irradiated ¼ in. corncob bedding (Envigo, Indianapolis, IN) and provided with approximately 8 g of paper nesting material (Enviro-Dri; Shepherd Specialty Papers, Watertown TN). Irradiated, pelleted rodent diet (Teklad Global Rodent Diet 2918; Envigo, Indianapolis, IN) and water bottles containing municipal water were provided ad libitum. The cages were placed on a stainless-steel rack in a room that was maintained at 20 to 23.3 °C (68 to 73.9 °F) and 30% to 70% relative humidity. The light:dark photoperiod was 14:10 with the lights turning on at 0600. The room was part of a vivarium that is managed by the Research Animal Resources department and covered by the AAALAC-accredited animal care and use program at the University of Minnesota.

Experimental design. The study and experimental procedures were approved by the University of Minnesota IACUC. The mice arrived in 2 separate cohorts, corresponding to the acute (n = 40) and chronic groups (n = 40). Within each group, mice were housed in pairs, and each cage of paired mice was randomly assigned to either dirty bedding (n = 20) or control (clean bedding) (n = 20). Because bedding treatments were administered at the cage level, the experimental unit was defined as the cage. The measurements for all experimental parameters were averaged for each pair of mice in a cage for both the dirty bedding (n = 10) and control (n = 10) treatments. At the time of arrival, 1 mouse in each cage was identified by a single ear punch. All cages were in the same room on 1 single-sided stainless-steel rack. To avoid unnecessary manipulation by other investigators or staff, no other mice were housed on the same rack. Placement of cages on the rack was randomized. Cages remained in the same position for the duration of the study. The mice were then allowed to acclimate to the environmental conditions for 7 d.

At the start of the experiment (day 0), mice were moved into a new cage containing their assigned bedding treatment. The mice in the dirty bedding treatment group were placed into a cage containing 100% dirty corncob bedding from colony mice whereas the control mice were placed into cages containing clean corncob bedding. All cages received new paper nesting material (Enviro-Dri; Shepherd Specialty Papers, Watertown TN), food (Teklad Global Rodent Diet 2918; Envigo, Indianapolis, IN), and water bottles. The acute groups of mice underwent only 1 cage change procedure (day 0) whereas the chronic groups experienced weekly cage changes for 4 wk (days 0, 7, 14, 21, 28). Cage change occurred between 1400 to 1600. Handling for this cage change procedure involved grasping the mouse at the base of their tail. Mice in the chronic groups were weighed on a digital scale during the cage change procedure between removal from the previous cage and transfer to the new cage. For each group of mice, 2 types of behavioral assessment (home cage and behavioral core tasks) were performed at certain time points. For the acute group, home cage assessment took place 1 d before (day -1) and 1 d after (day 1) cage change. Behavioral core assessment occurred 1 day after cage change (day 1). For the chronic group, home cage assessment took place 1 d before (days -1, 6, 13, 20, 27) and 1 d after (days 1, 8, 15, 22, 29) cage changes. On the day after the last cage change (day 29), the chronic group of mice also underwent behavioral core testing. At the end of the study (day 1 for the acute group; day 29 for the chronic group) after completion of all the behavioral tests, each mouse was euthanized using CO_2 and blood and vaginal cytology samples were collected for leukocyte and estrous cycle analysis, respectively.

Dirty bedding. Dirty bedding was collected from colony mice housed in the same room as the experimental cages. Colony mice were housed under similar conditions as the experimental mice in static microisolation cages containing irradiated corncob bedding and paper nesting material. Irradiated, pelleted rodent diet, and water bottles containing municipal water were provided ad libitum. Colony mice were free of the following agents: Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvovirus, Theiler murine encephalomyelitis virus, reovirus, murine rotavirus, lymphocytic choriomeningitis virus, ectromelia virus, mouse adenovirus, mouse cytomegalovirus, polyoma virus, *Mycoplasma pulmonis*, *Clostridium piliforme*, cilia-associated respiratory bacillus, *Encephalitozoon cuniculi*, fur mites, and pinworms.

The colony mice were being used on multiple IACUC-approved protocols held by different principal investigators. The colony mice consisted of a mix of male and female mice on experiment (both singly and grouped housed) as well as breeding animals. Multiple strains, including genetically modified mice, were also present. Per the IACUC protocols, none of the colony mice were expected to have any abnormal gross changes to their feces or urine (for example melena, diarrhea, hematuria). On the day of cage change, all the dirty cages from the room (200 to 250 depending on the day) were collected. A portion of the dirty bedding (approximately 40 g) from every cage was removed and pooled into a single container. The dirty

bedding mainly consisted of the corncob bedding and feces, but some also contained ground food pellets. Nesting material was excluded. Once dirty bedding was collected from all the colony cages, it was manually mixed for 5 min. To create the dirty bedding treatment cages, the combined dirty bedding was added to the bottom of a clean cage to a depth of 1/4 inch (approximately 200 g). New nesting material was then placed in one corner of the cage. The cages were completed by providing fresh food and water in the wire top and a microisolation cage lid.

Home cage behavioral assessments. Home cage assessment consisted of scoring of the hair coat and nest and observing for stereotypical behavior. The hair coat of each mouse was assigned a score based on its appearance (0: shiny, smooth, well-kept hair coat; 1: at least one area of the body with rough hair). A previously described hair coat scoring system was modified to allow for cage-side assessment and avoid manipulation of the mice.[30] One nest score was assigned per cage (0: no manipulation of nest material; 1: no discernable nest; 2: flat nest with no shallow walls; 3: nest with a slightly cupped shape with walls less than half the height of a dome that would cover a mouse; 4: nest with walls that are half the height of a dome; 5: nest with walls greater than half the height of a dome which may or may not fully enclose the nest) as previously described.[32] To assess stereotypical behavior, each mouse was observed for 5 continuous minutes for the presence of stereotypies (bar-mouthing, circling, twirling, back-flipping, route-tracing) based on previous descriptions.[64] Behavior was scored (0: absent; 1: 3 unbroken repeats of the same stereotypical behavior [circling, twirling, back-flipping, route-tracing] or repeated continuously for 3 s [bar-mouthing]) and the type of stereotypical behavior was recorded when present. Home cage behavioral assessments were performed during the last 2 h of the dark cycle (0400-0600). One observer performed all home cage assessments. Scoring was performed on the rack without moving or disturbing the cage using a red-light flashlight. If an assessment could not be made due to poor visibility, a score was not recorded.

Core behavioral task assessments. The University of Minnesota Mouse Behavior Core provided guidance and support on behavioral tasks performed in their facility. Mice were moved from housing to the testing facility and were allowed to acclimate for 1 h prior to testing. Mice underwent sequential behavioral tests consisting of light-dark box followed by elevated plus maze testing. Both mice in a cage were subjected to the light-dark box test simultaneously using 2 identical testing apparatuses. After completion of the light-dark box testing, one mouse from the cage was immediately subjected to elevated plus maze assessment while the other mouse was returned to the cage. The other mouse was tested on the elevated plus maze after the first mouse had completed the test. All equipment was cleaned with 70% ethanol between mice. Testing occurred between 0900 to 1500. One test operator performed all behavior core testing. The cage testing order was randomized.

The light-dark box consisted of a modified polycarbonate cage (48.26 cm long x 25.4 cm wide x 20.32 cm tall, Allentown) split into a light side (32.16 cm long x 25.4 cm wide) and a dark side (16.10 cm long x 25.4 cm wide) by a dividing wall with a small portal (3.81 cm tall x 6.35 cm wide) at the base. The light chamber contained overhead illumination (1400 lx). At the beginning of the test, the mice were placed in the light chamber. The mice were subsequently recorded using an overhead digital camera for 10 min. Two observers who were blind to the mouse treatment group independently viewed the recorded footage and scored the overall time spent in the light chamber (s) and latency to enter the dark chamber (s).

The elevated plus maze (Med-Associated; St. Albans, VT) consisted of 2 open arms (34.9 cm long x 6.07 cm wide) intersecting with 2 closed arms (34.9 cm long x 6.07 cm wide x 19.13 cm tall). The entire apparatus was elevated (74.93 cm above base) and dimly illuminated (45 lx) by overhead lights. At the beginning of the test, the mice were placed in the center (2.4 long x 2.4 cm wide) of the intersecting arms. The mice were subsequently recorded using an overhead digital camera for 5 min. Two observers who were blind to the treatment group independently viewed the recorded footage and scored the amount of time spent in the open arms (s). The proportion of time spent in the open arms was calculated as the time in open arms divided by the sum of time in both the open arms and closed arms.

Postmortem collection of samples. Immediately after the completion of the behavior core testing (day 1 for the acute group; day 29 for the chronic group), the mice were placed back into their home cage and the pair was euthanized using CO_2. Euthanasia was performed in the home cage using a displacement rate of 30 – 70% of cage volume per minute. After confirmation of death, approximately 0.5 mL of whole blood was collected by cardiac puncture. The blood was immediately placed in an EDTA collection tube (K2EDTA MiniCollect Tube; Greiner Bio-One, Kremsmünster, Austria). The tubes were gently inverted for 10 s and then placed on ice until shipment. Two personnel collected blood (one person for each mouse) to ensure rapid collection of unclotted blood. After blood collection, a sterile swab (Sterile Polyester Tipped Applicators 25 to 826 2WD; Puritan, Guilford, ME) was wet with sterile saline (0.9% NaCl) and inserted into the vagina of each mouse. The swab was gently turned and rolled against the vaginal wall and then removed. Cells were transferred to a dry glass slide by rolling the swab across the slide. The slides were allowed to air-dry for at least 30 min. Sample collection for vaginal cytology was performed by one person.

Complete blood count. On the same day of collection, the blood samples were shipped next-day on ice to IDEXX Reference Laboratory (Grafton, MA). Complete blood count testing was performed on every sample using a commercial analyzer (XT-V Analyzer; Sysmex America, Lincolnshire, IL). A technician reviewed blood smears microscopically for abnormalities. The reported absolute neutrophil and lymphocyte numbers (/uL) were used to calculate the NLR for each sample.

Vaginal cytology. The air-dried slides were stained with a Romanowsky-type stain (Rapid Differential Stain Kit; VetOne, Boise, ID) according to the manufacturer's instructions. The slides were examined microscopically, and the stage of the estrous cycle was determined for each mouse as described previously.[16] One observer who was blind to the treatment group determined the estrous cycle stage of each mouse.

Statistics. To assess the agreement of the 2 observers for the light-dark box and elevated plus maze, intraclass correlations (ICC) were calculated and mean difference plots were created. To analyze the difference between bedding treatment, all behavioral and physiologic measurements were averaged for both mice in the same cage to account for the application of the treatment at the cage level and not to individual mice. Using these cage averages, *t* tests were used to compare the following data by treatment group: nest score, light-dark box (time spent in the light chamber, latency to enter the dark chamber), elevated plus maze (proportion of time spent in the open arm), NLR, and weight (by day, net weight gain). The acute and chronic groups were assessed separately. The haircoat scores were not analyzed because all the mice maintained a shiny, smooth, and well-kept hair coat at all time points regardless of treatment. The

order of testing in the elevated plus maze and the estrous cycle stage were assessed as covariates to adjust for any differences. A linear mixed model analysis was performed on each measure, with treatment as the main predictor, estrous stage and testing order as covariates, and a random effect for cage. All statistical analyses were performed using statistical software (R version 4.0.2.; Foundation for Statistical Computing, Vienna, Austria). The threshold for significance in all statistical tests was set at a P value of less than 0.05. Descriptive values are presented as mean ± 1 SD.

Results

Home cage behavior. For both the acute and chronic groups, most mice were visible for home cage assessment of hair coat and behavior at every time point. On average 7 of 40 mice from each group could not be assessed at each time point due to poor visibility. All mice in both the acute and chronic groups maintained a shiny, smooth, and well-kept hair coat at all time points regardless of treatment. No stereotypical behaviors were noted in the mice in the acute group. In the chronic groups, stereotypical behavior was noted in mice exposed to control and dirty bedding treatments. One control mouse was noted bar-mouthing (day 29). One mouse exposed to dirty bedding was identified as bar-mouthing (days 13 and 22) and another mouse was twirling (days 20, 22, and 29). The prevalence of these behaviors was too low to be analyzed statistically. No significant differences ($P < 0.05$) in nest scores based on treatment were noted in either the acute or chronic groups at any of the time points (Table 1).

Light-dark box and elevated plus maze. Data analyzed for the light-dark box included latency to enter the dark chamber and overall time spent in the light chamber (Table 1). There were no significant differences ($P < 0.05$) in these measurements between the control and dirty bedding treatments in either the acute or chronic groups (Figure 1A and B). There was little variance between reviewer scoring (ICC: 0.97 to 0.99).

For the elevated plus maze, the proportion of time spent in the open arm was calculated (Table 1). There was no significant difference ($P < 0.05$) between the control and dirty bedding treatments in either the acute or chronic groups (Figure 2). Variance between reviewers was mild (ICC: 0.83). No significant difference ($P < 0.05$) in the behavioral test measurements was detected between the mice that had elevated plus maze testing immediately and those that were delayed.

Neutrophil:lymphocyte ratio. One sample from a mouse exposed to control bedding in the acute group coagulated prior to analysis and white blood cell counts could not be obtained. The NLRs were calculated from the absolute leukocyte counts (Table 1). No significant differences ($P < 0.05$) were detected in the NLR between treatments in either the acute or chronic groups (Figure 3). No significant difference ($P < 0.05$) in the NLR was detected between mice that underwent elevated plus maze testing immediately and those that were delayed.

Weight. Because weight changes were considered unlikely in the acute groups, weights were collected only for mice in the chronic groups (Table 1). Mice that were exposed chronically to dirty bedding weighed significantly less than mice in the control group at day 21 (27.6 ± 1.6 g and 29.4 ± 1.0 g,

Table 1. Mean ± SD for behavioral and physiologic parameters measured in the acute and chronic groups treated with control or dirty bedding.

	Acute		Chronic	
	Control	Dirty Bedding	Control	Dirty Bedding
Nest Scores				
D-1	3.4 ± 1.1	3.5 ± 0.9	3.8 ± 0.9	4.0 ± 1.2
D1	3.7 ± 0.8	3.2 ± 0.9	3.5 ± 1.0	2.6 ± 0.5
D6			3.2 ± 0.9	3.8 ± 0.8
D8			3.5 ± 1.4	3.3 ± 0.8
D13			3.8 ± 0.9	2.9 ± 0.9
D15			3.4 ± 1.3	3.6 ± 1.0
D20			3.8 ± 1.4	3.5 ± 1.2
D22			3.5 ± 1.1	3.4 ± 1.2
D27			3.2 ± 0.9	2.9 ± 0.7
D29			3.9 ± 1.1	3.6 ± 1.4
Light-Dark Box				
Time Spent in Light Chamber (s)	125 ± 66	174 ± 81	100 ± 55	142 ± 74
Latency to Enter Dark Chamber (s)	27 ± 21	36 ± 26	34 ± 34	29 ± 22
Elevated Plus Maze				
Proportion of Time Spent in the Open Arm	0.42 ± 0.12	0.36 ± 0.10	0.41 ± 0.11	0.40 ± 0.07
Neutrophil:Lymphocyte Ratio	0.17 ± 0.03	0.17 ± 0.04	0.12 ± 0.05	0.14 ± 0.04
Weight				
D0 (g)			24.45 ± 1.04	25.15 ± 0.91
D7 (g)			25.75 ± 0.92	25.50 ± 1.08
D14 (g)			27.40 ± 1.07	26.55 ± 1.32
D21 (g)			29.35 ± 0.97	27.55 ± 1.61 *
D28 (g)			31.10 ± 1.22	28.70 ± 1.57 *
Net Gain (g)			6.65 ± 1.53	3.55 ± 0.90 *

*$P < 0.05$

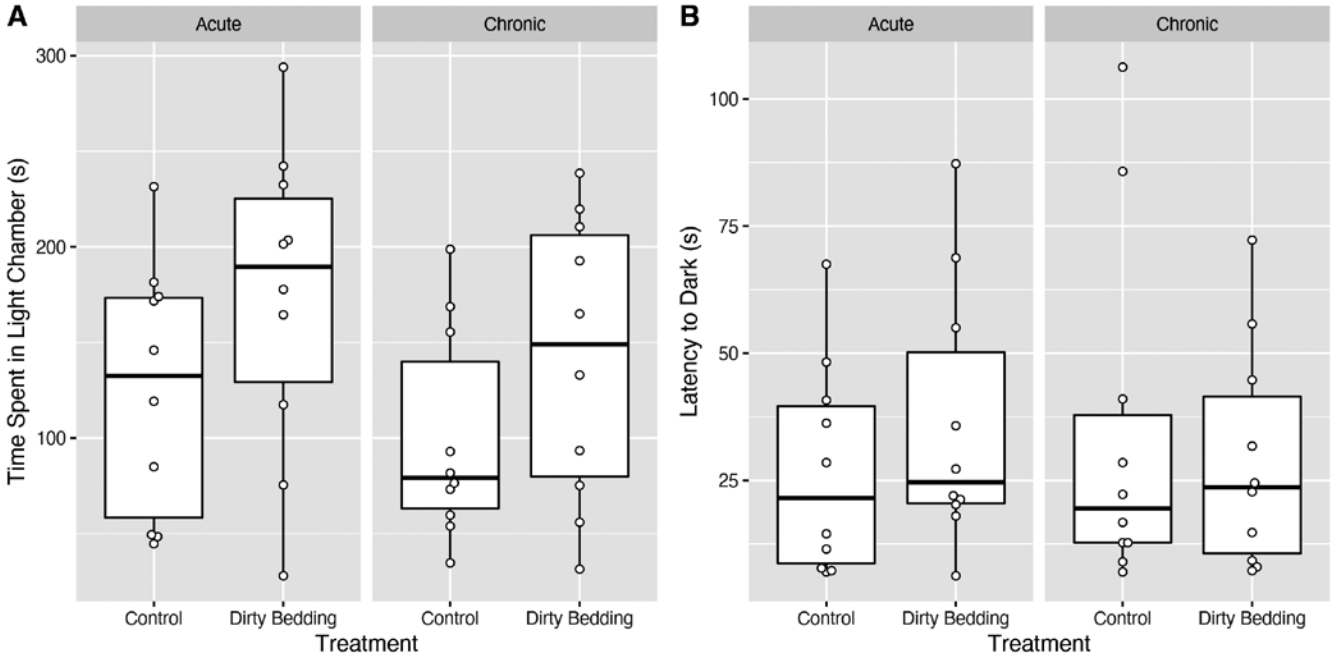

Figure 1. Results of the light-dark box behavioral assay for acute and chronic groups exposed to control or dirty bedding. (A) Box and whisker plot with solid black line representing the median amount of time spent in the light chamber (s). (B) Box and whisker plot with solid black line representing the median latency to enter the dark chamber (s). No significant differences between control or dirty bedding were noted (n = 10 per treatment, t test).

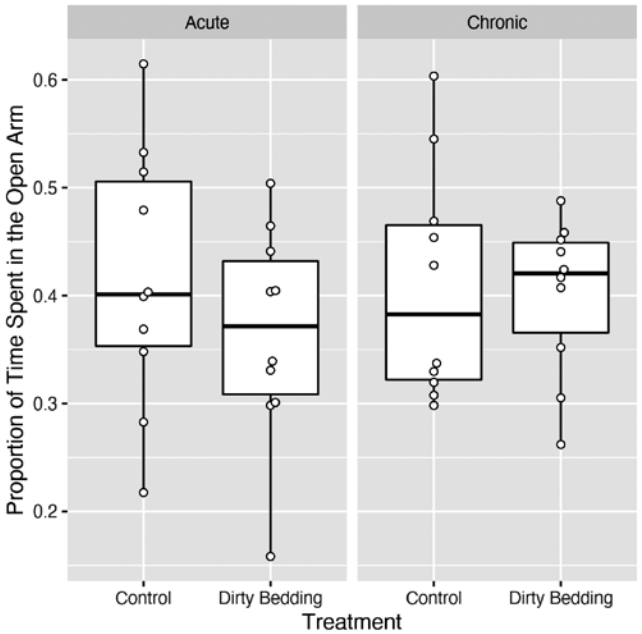

Figure 2. Results of the elevated plus maze behavioral assay for acute and chronic groups exposed to control or dirty bedding. Box and whisker plot with the solid black line representing the median proportion of time spent in the open arms. No significant differences between control or dirty bedding were noted (n = 10 per treatment, t test).

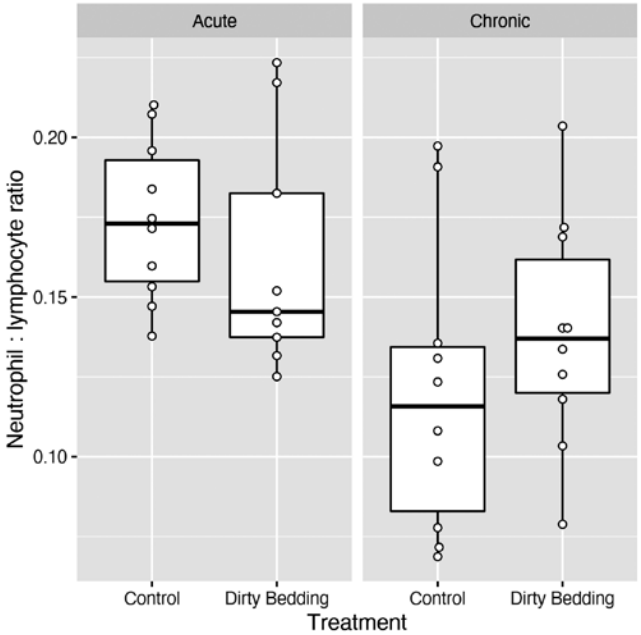

Figure 3. Neutrophil:lymphocyte ratio for acute and chronic groups exposed to control or dirty bedding. Box and whisker plot with the solid black line representing the median neutrophil:lymphocyte ratio. No significant differences between control or dirty bedding were noted (n = 10 per treatment, t test).

respectively; P = 0.009) and day 28 (28.7 ± 1.6 g and 31.1 ± 1.2 g, respectively; P = 0.001). (Figure 4B). The net change in weight over the entire study was also significantly less for mice exposed chronically to dirty bedding as compared with control mice (3.6 ± 0.9 g compared with 6.7 ± 1.5 g, respectively; P = 0.00006) (Figure 4A).

Estrous cycle. Estrous cycle stage as a covariate did not have any significant impact on any of the measurements.

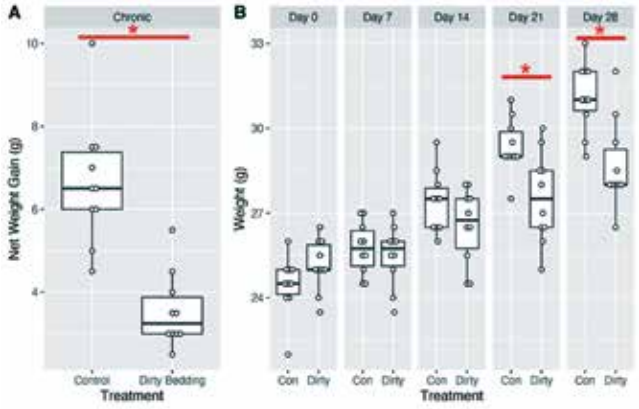

Figure 4. Weights of mice chronically exposed to control or dirty bedding. (A) Box and whisker plot with the solid black line representing the median weight gain (g) over the study duration. (B) Box and whisker plot with the solid black line representing the median weight (g) per day. Significance is set at $p < 0.05$ and is indicated by * ($n = 10$ per treatment, t test).

Discussion

Dirty-bedding sentinels are regularly exposed to soiled bedding from colony animals to transfer and detect excluded microorganisms in the resident population. However, dirty bedding can also contain other elements, such as pheromones and ammonia, which may act as a source of stress for sentinel mice. The goal of this study was to determine whether acute or chronic dirty bedding exposure causes behavioral and/or physiologic changes consistent with stress to better understand the experience of a sentinel mouse.

No significant differences in home cage behavior (hair coat, nest score, stereotypical behavior), behavioral tests (light-dark box and elevated plus maze), or NLR were detected between the mice exposed to dirty or clean bedding in the acute or chronic groups. However, the mice exposed to dirty bedding in the chronic group weighed significantly less at days 21 and 28 as compared with the control mice. The chronic dirty bedding mice also had a significantly lower net weight gain over the course of the study.

Stress describes an internal state that occurs in response to a disturbance in homeostasis or wellbeing.[62] Stress in animals must be inferred from behavioral and physiologic responses to stressors. One method of assessing stress is by identifying significant deviations from species-specific behavior.[60] Self-grooming and nest building are common natural behaviors in mice that can be used to identify changes consistent with stress.[30,32,45] In this study, no significant differences in hair coat or nest scores were detected between the mice exposed to dirty or control bedding. All the mice in both the acute and chronic groups maintained a well-kept hair coat with no areas of rough hair to indicate a lack of grooming. Nest scores of the acute groups ranged from 2-5 on all days measured regardless of treatment. For the chronic groups, only the control group measured on day 1 showed a tighter range of 3-5. At all of the other timepoints, the nest scores ranged from 2-5 regardless of treatment. Others have studied grooming and nest building over time, which may provide more sensitive assessment of behavioral alterations.[73,90] This was not done in the present study due to the desire to maintain consistent timing of treatment and observations in each group of mice, and because of lack of enough equipment for observation of 40 cages of mice simultaneously.

Another method of assessing stress is by identifying abnormal behaviors.[19] Stereotypical behaviors are classified as abnormal repetitive behaviors that lack any goal or function.[31] They are complex behaviors that are not well understood, but have been suggested to be associated with stress although a true cause and effect relationship has not been established.[26,93] In this study, mice chronically exposed to control (1 of 20) and dirty bedding (2 of 20) showed stereotypical behavior during the observation period. Stereotypical behaviors were noted earlier in the mice exposed to dirty bedding than in the control mice (on days 13 and 20 as compared with day 29). In individual mice that showed stereotypical behavior on multiple observations throughout the study, the presence of the abnormal behavior was not observed consistently after first being identified. Based on the literature, the prevalence of stereotypical behavior was lower than expected for this stock of mice.[31,94] As for grooming and nest building, stereotypical behavior can vary over time such that investigators observe mice during the entire dark phase to capture these behaviors.[63,64] This variation may be present in our data given that the behaviors were intermittent once identified. This variation may also account for the unexpectedly low prevalence of stereotypical behaviors.[94] Given that the prevalence of stereotypical behaviors was too low to analyze, increasing the duration of observation may have yielded more instances of stereotypy. This was not done in our study due to an effort to maintain consistencies in the timing of treatments and insufficient equipment to monitor this number of mice simultaneously as stated above.

In humans, stress, especially when prolonged or repeated, can lead to emotional disturbances including anxiety and depression.[15] Therefore, another method of assessing stress is identifying these emotional changes. Several well-established behavioral tests in mice are used to assess anxiety.[2,81] A battery of tests is often recommended to define the behavioral phenotype. The light-dark box and elevated plus maze are 2 of the most widely used tests for anxiety that rely on the conflict between exploration and avoidance.[2] Mice prefer dark, closed areas instead of light, open spaces, so decreased willingness to explore environments that are more aversive is used to identify anxiety. Based on our data, exposure to dirty bedding (acute or chronic) did not significantly affect any of the parameters measured in the light-dark box or elevated plus maze. Agreement of the 2 reviewers varied from very good (light-dark box) to moderate (elevated plus maze). Possible causes for the variation in viewer agreement for the elevated plus maze include the complexity of the apparatus and/or visualization of the mice. The elevated plus maze has 3 areas - open arms, closed arms, and central area. The presence of the central area may have contributed to discrepancies between the reviewers. In addition, the apparatus used was built for mice with black coats so that the observer could benefit from the contrast of a dark mouse on a light background. However, the CD-1 mice used in this study are white. The lack of contrast between the white hair coats and white background may have added a layer of complexity to the assessment.

The majority of laboratory tests for anxiety-like behavior are validated and performed with male rodents despite evidence for sex-related differences in stress psychopathology.[7,53] For our study, the light-dark box and elevated plus maze were used because they take advantage of characteristic behaviors (that is avoidance of light or open areas) that are present in both sexes, despite some minor differences.[66] Other behavioral tests of anxiety and stress can have large differences between female and male responses, which can confuse interpretation of results.[67] This is especially true for assessments of psychosocial stress, which often involve male dominance and aggression.[4,67]

Because dirty bedding is postulated to act as a source of olfactory-mediated psychosocial stress, the use of other behavioral assessments in this study may have shown significant results; however, the light-dark box and elevated plus maze minimize potential sex-related difficulties in test results.

In addition to behavioral changes, stressful stimuli can also result in characteristic physiologic changes, including alterations to the endocrine,[54,56] nervous,[33] immune,[20,23,84] and reproductive systems.[86] One such change is activation of the hypothalamic-pituitary-adrenal (HPA) axis and the production of glucocorticoids, including cortisol and corticosterone.[56] In response to stress, the hypothalamus synthesizes and releases corticotropin-releasing hormone, which stimulates the pituitary to release adrenocorticotropic hormone. Adrenocorticotropic hormone acts to promote the release of glucocorticoids by the adrenal glands. Because of this system, glucocorticoid levels have been used experimentally to quantify the impact of stressful stimuli.[80] Despite its widespread use, several limitations can impact data results and interpretation, including circadian fluctuations and pulsatile secretion rhythms.[80] In addition, many sampling and experimental procedures cause significant elevations in glucocorticoids, which may obscure meaningful results.[80] Due to these limitations, other measurements of the HPA axis have been investigated. One measurement is NLR.[18,27,35] Glucocorticoids mediate an increase in the number of neutrophils and a decrease in lymphocytes, causing an increase in the NLR. Furthermore, because this change occurs more slowly, it has the potential to eliminate confounding increases due to experimental procedures, such as the behavioral tests that were run in this study. No significant differences were detected in the NLR of mice exposed to dirty or control bedding in either the acute or chronic group. No evidence of increased lymphocytes was found in response to antigens in the dirty bedding, which could have confounded the interpretation of this ratio. Not all stressors activate the HPA axis, so other measurements may have resulted in significant effects.

Changes in body weight have also been identified as a consequence of stress in laboratory rodents. Depending on sex and the type of experimental procedure, stress can result in either body weight gain or weight loss.[17,39,70,77,79,89] Decreased body weight is generally considered indicative of stress, especially when bolstered by other behavioral and physiologic changes.[70] In this study, the mice that were chronically exposed to dirty bedding had a significantly lower body weight on days 21 and 28 and decreased overall weight gain as compared with control mice. This significant effect was identified after multiple exposures to dirty bedding. However, despite this difference, all mice remained within the expected weight range based on age as provided by the vendor (Charles River Laboratories). The neurophysiological mechanisms of stress-induced negative energy balance are still being elucidated, but likely involve both central and peripheral changes.[70,71] These changes are mediated not only by glucocorticoids, but also by other factors including but not limited to leptin and catecholamines, which induce changes to food intake and lipolysis, respectively. In addition, mounting evidence suggests that the composition of the gut microbiome plays a significant role in metabolism and the development of lean or obese phenotypes.[40,61] The responsible mechanisms are complex and still being identified, but may include microbiota-induced changes in nutrient breakdown and utilization,[85] regulation of adipose tissue,[1] and secretion of bacterial products resulting in inflammatory[21] and hormonal changes.[78] Therefore, exposure to dirty bedding and subsequent gut microbiome changes could be responsible for decreased weight gain independent of stress. Identification of the metabolic changes in response to dirty bedding was beyond the scope of this study, but considering the results, further studies could establish the specific mechanism(s) and determine how they may or may not relate to known stress-related energy balance deviations.

The estrous cycle stage is an important experimental variable in female mice and must be considered when interpreting results. Fluctuations in sex hormones (estrogen and progesterone) can result in significant differences in the interpretation of stress- and anxiety-related behavioral tests and physiologic responses.[67,83] In this study, the stage of the estrous cycle was determined by vaginal swabs performed postmortem. The stage of the estrous cycle did not significantly affect any of the measures. Mice in both the acute and chronic groups were in various stages of the estrous cycle, with each stage found at least once in both treatment groups. Despite our negative data, the potential role of the estrous cycle stage as a variable warrants future study into the effects of dirty bedding on reproduction. Mice were not sexually mature at the beginning of the study, making them particularly vulnerable to the effects of sex pheromones that can accelerate[87] or delay maturation.[24] Nutritional status and body weight may be associated with the onset of sexual maturity. In mice, the relationship between body weight and puberty is complex and an area of current study, but available evidence suggests that low body weight is associated with delayed sexual maturation.[5,28] Given the potential reproductive effects of pheromone-containing dirty bedding and the age of the mice, the age of sexual maturation and estrous cycling could be measured in future experiments with regard to the cause of the decreased weight gain. In addition, stress is known to also affect sexual development.[25,52] Therefore, further investigation could help to determine whether the weight changes observed in this study were related to stress. Monitoring sexual maturation and the estrous cycle requires regular handling and therefore was not performed in the current study to avoid disruption associated with visual examination and/or sampling.

Stress in the laboratory setting can be defined by a multitude of behavioral and physiologic tests and predicting which tests will provide significant results based on the stressor is difficult. In the current study, the tests were chosen for reasons related to the sex of the mice, the characteristics of the stressor, timeline of exposure, and potential interactions between tests. Other tests that may have yielded significant results and helped to define the complete phenotype that results from dirty bedding exposure were outside the scope of this study.

Another important limitation is the variable nature of dirty bedding. Depending on the donor mice and their use in research, dirty bedding can be quite diverse with regard to its composition and the presence of potential stressors (for example pheromones, ammonia). Therefore, depending on the composition of the dirty bedding, individual sentinel mice may be exposed to different levels of stressors. In the current study, this variation was intentionally minimized, but not eliminated, by thoroughly mixing bedding prior to exposure, but the choice of a different source of colony mice could yield different results.

To summarize, mice that were exposed chronically to dirty bedding weighed less in the latter part of the study (days 21 and 28) and had an overall lower net weight gain over the 28-day duration of the study. Increasing the duration of exposure could provide additional data that assists in determining whether exposure to dirty bedding is associated with stress. Because sentinel mice are often exposed to dirty bedding for much longer time periods (for example 6 to 12 mo), increasing the duration

of exposure could be the focus of a future study. In addition, studying the mechanisms of this difference in weight (for example food consumption, body mass analysis, levels of metabolic hormones and neurotransmitters, microbiome, estrous cycle, sexual maturity) to identify the cause(s) of this change could further elucidate the effects of dirty bedding exposure. Understanding the effects of prolonged dirty bedding exposure and the specific pathways that are modulated could help to further define and improve the housing of sentinel mice.

Acknowledgments

The authors thank Max Meyers, Danielle Hyde, and Sarah Muellner of Research Animal Resources for their assistance organizing the husbandry aspects of this experiment, including the housing of the animals, coordination of cage change and the collection of dirty cages. The authors would also like to thank Erin Larsen, Orion Rainwater, and Michael Benneyworth of the University of Minnesota's Mouse Behavior Core for their assistance in planning and running the behavioral tests. Lastly, the authors thank Nick Burrows and Britta Gauer of Research Animal Resources for their technical assistance collecting post-mortem samples.

References

1. **Bäckhed F, Ding H, Wang T, Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI.** 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci USA **101:**15718–15723. https://doi.org/10.1073/pnas.0407076101.
2. **Bailey KR, Crawley JN.** 2009. Anxiety-related behaviors in mice. In: Buccafusco JJ, editor. Methods of behavior analysis in neuroscience, 2nd ed. Boca Raton (FL): CRC Press/Taylor & Francis.
3. **Bind RH, Minney SM, Rosenfeld S, Hallock RM.** 2013. The role of pheromonal responses in rodent behavior: future directions for the development of laboratory protocols. J Am Assoc Lab Anim Sci **52:**124–129.
4. **Blanchard RJ, McKittrick CR, Blanchard DC.** 2001. Animal models of social stress: effects on behavior and brain neurochemical systems. Physiol Behav **73:**261–271. https://doi.org/10.1016/S0031-9384(01)00449-8.
5. **Bohlen TM, Silveira MA, Zampieri TT, Frazão R, Donato J Jr.** 2016. Fatness rather than leptin sensitivity determines the timing of puberty in female mice. Mol Cell Endocrinol **423:**11–21. https://doi.org/10.1016/j.mce.2015.12.022.
6. **Boillat M, Challet L, Rossier D, Kan C, Carleton A, Rodriguez I.** 2015. The vomeronasal system mediates sick conspecific avoidance. Curr Biol **25:**251–255. https://doi.org/10.1016/j.cub.2014.11.061.
7. **Bolea-Alamanac B, Bailey SJ, Lovick TA, Scheele D, Valentino R.** 2018. Female psychopharmacology matters! Towards a sex-specific psychopharmacology. J Psychopharmacol **32:**125–133. https://doi.org/10.1177/0269881117747578.
8. **Bonaventure P, Yun S, Johnson PL, Shekhar A, Fitz SD, Shireman BT, Lebold TP, Nepomuceno D, Lord B, Wennerholm M, Shelton J, Carruthers N, Lovenberg T, Dugovic C.** 2015. A selective orexin-1 receptor antagonist attenuates stress-induced hyperarousal without hypnotic effects. J Pharmacol Exp Ther **352:**590–601. https://doi.org/10.1124/jpet.114.220392.
9. **Brechbühl J, Moine F, Klaey M, Nenniger-Tosato M, Hurni N, Sporkert F, Giroud C, Broillet MC.** 2013. Mouse alarm pheromone shares structural similarity with predator scents. Proc Natl Acad Sci USA **110:**4762–4767. https://doi.org/10.1073/pnas.1214249110.
10. **Bruce HM.** 1959. An exteroceptive block to pregnancy in the mouse. Nature **184:**105. https://doi.org/10.1038/184105a0.
11. **Bruce HM.** 1970. Pheromones. Br Med Bull **26:**10–13. https://doi.org/10.1093/oxfordjournals.bmb.a070734.
12. **Buckley LA, Jiang XZ, James RA, Morgan KT, Barrow CS.** 1984. Respiratory-tract lesions induced by sensory irritants at the Rd50 concentration. Toxicol Appl Pharmacol **74:**417–429. https://doi.org/10.1016/0041-008X(84)90295-3.
13. **Cantara SI, Blas-Machado U, Zhao X, Moore RH, Schroeder JP, Lee VK.** 2020. Comparative effects of 1/4-inch and 1/8-inch corncob bedding on cage ammonia levels, behavior, and respiratory pathology of male C57BL/6 and 129S1/Svlm mice. J Am Assoc Lab Anim Sci **59:**695–702. https://doi.org/10.30802/AALAS-JAALAS-19-000152.
14. **Chamero P, Marton TF, Logan DW, Flanagan K, Cruz JR, Saghatelian A, Cravatt BF, Stowers L.** 2007. Identification of protein pheromones that promote aggressive behaviour. Nature **450:**899–902. https://doi.org/10.1038/nature05997.
15. **Calabrese F, Molteni R, Racagini G, Riva MA.** 2009. Neuronal plasticity: a link between stress and mood disorders. Pyschoneuroendocrinology. **34S:**S208–216. https://doi.org/10.1016/j.psyneuen.2009.05.014.
16. **Cora MC, Kooistra L, Travlos G.** 2015. Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. Toxicol Pathol **43:**776–793. https://doi.org/10.1177/0192623315570339.
17. **Dadomo H, Gioiosa L, Cigalotti J, Ceresini G, Parmigiani S, Palanza P.** 2018. What is stressful for females? Differential effects of unpredictable environmental or social stress in CD1 female mice. Horm Behav **98:**22–32. https://doi.org/10.1016/j.yhbeh.2017.11.013.
18. **Davis A, Maney D, Maerz J.** 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol **22:**760–772. https://doi.org/10.1111/j.1365-2435.2008.01467.x.
19. **Dawkins MS.** 1990. From an animal's point of view: Motivation, fitness, and animal welfare. Behav Brain Sci **13:**1–9. https://doi.org/10.1017/S0140525X00077104.
20. **Deak T, Nguyen KT, Fleshner M, Watkins LR, Maier SF.** 1999. Acute stress may facilitate recovery from a subcutaneous bacterial challenge. Neuroimmunomodulation **6:**344–354. https://doi.org/10.1159/000026394.
21. **Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NM, Magness S, Jobin C, Lund PK.** 2010. High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. PLoS One **5:**e12191. https://doi.org/10.1371/journal.pone.0012191.
22. **Domer DA, Erickson RL, Petty JM, Bergdall VK, Hickman-Davis JM.** 2012. Processing and treatment of corncob bedding affects cage-change frequency for C57BL/6 mice. J Am Assoc Lab Anim Sci **51:**162–169.
23. **Dragoş D, Tănăsescu MD.** 2010. The effect of stress on the defense systems. J Med Life **3:**10–18.
24. **Drickamer LC.** 1974. Sexual maturation of female house mice: social inhibition. Dev Psychobiol **7:**257–265. https://doi.org/10.1002/dev.420070310.
25. **Eck SR, Ardekani CS, Salvatore M, Luz S, Kim ED, Rogers CM, Hall A, Lee DE, Famularo ST, Bhatnagar S, Bangasser DA.** 2019. The effects of early life adversity on growth, maturation, and steroid hormones in male and female rats. Eur J Neurosci **52:**2664–2680. https://doi.org/10.1111/ejn.14609.
26. **Engel AKJ, Gross AN, Richterc SH, Rommen J, Toumad C, Würbel H.** 2011. Variation in stress reactivity affects cage-induced stereotypies in female CD-1 (ICR) mice. Appl Anim Behav Sci **133:**101–108. https://doi.org/10.1016/j.applanim.2011.04.017.
27. **Everds NE, Snyder PW, Bailey KL, Bolon B, Creasy DM, Foley GL, Rosol TJ, Sellers T.** 2013. Interpreting stress responses during routine toxicity studies: a review of the biology, impact, and assessment. Toxicol Pathol **41:**560–614. https://doi.org/10.1177/0192623312466452.
28. **Falconer DS.** 1984. Weight and age at puberty in female and male mice of strains selected for large and small body size. Genet Res **44:**47–72. https://doi.org/10.1017/S0016672300026240.
29. **Ferrecchia CE, Jensen K, Van Andel R.** 2014. Intracage ammonia levels in static and individually ventilated cages housing C57BL/6 mice on 4 bedding substrates. J Am Assoc Lab Anim Sci **53:**146–151.
30. **Frisbee JC, Brooks SD, Stanley SC, d'Audiffret AC.** 2015. An unpredictable chronic mild stress protocol for instigating depressive symptoms, behavioral changes and negative health outcomes in rodents. J Vis Exp **106:**53109. https://doi.org/10.3791/53109.
31. **Garner JP.** 2005. Stereotypies and other abnormal repetitive behaviors: potential impact on validity, reliability, and replicability of

scientific outcomes. ILAR J **46:**106–117. https://doi.org/10.1093/ilar.46.2.106.

32. **Gaskill BN, Karas AZ, Garner JP, Pritchett-Corning KR.** 2013. Nest building as an indicator of health and welfare in laboratory mice. J Vis Exp **82:**51012. https://doi.org/10.3791/51012.
33. **Goldstein DS.** 1987. Stress-induced activation of the sympathetic nervous system. Baillieres Clin Endocrinol Metab **1:**253–278. https://doi.org/10.1016/S0950-351X(87)80063-0.
34. **Haga S, Hattori T, Sato T, Sato K, Matsuda S, Kobayakawa R, Sakano H, Yoshihara Y, Kikusui T, Touhara K.** 2010. The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. Nature **466:**118–122. https://doi.org/10.1038/nature09142.
35. **Hickman DL.** 2017. Evaluation of the neutrophil:lymphocyte ratio as an indicator of chronic distress in the laboratory mouse. Lab Anim (NY) **46:**303–307. https://doi.org/10.1038/laban.1298.
36. **Hoover JE, Drickamer LC.** 1979. Effects of urine from pregnant and lactating female house mice on oestrous cycles of adult females. J Reprod Fertil **55:**297–301. https://doi.org/10.1530/jrf.0.0550297.
37. **Horn MJ, Hudson SV, Bostrom LA, Cooper DM.** 2012. Effects of cage density, sanitation frequency, and bedding type on animal wellbeing and health and cage environment in mice and rats. J Am Assoc Lab Anim Sci **51:**781–788.
38. **Inagaki H, Kiyokawa Y, Kikusui T, Takeuchi Y, Mori Y.** 2008. Enhancement of the acoustic startle reflex by an alarm pheromone in male rats. Physiol Behav **93:**606–611. https://doi.org/10.1016/j.physbeh.2007.10.021.
39. **Karamihalev S, Brivio E, Flachskamm C, Stoffel R, Schmidt MV, Chen A.** 2020. Social dominance mediates behavioral adaptation to chronic stress in a sex-specific manner. eLife **9:**e58723. https://doi.org/10.7554/eLife.58723.
40. **Khan MJ, Gerasimidis K, Edwards CA, Shaikh MG.** 2016. Role of gut microbiota in the aetiology of obesity: proposed mechanisms and review of the literature. J Obes **2016:** 1–27. https://doi.org/10.1155/2016/7353642.
41. **Kimoto H, Haga S, Sato K, Touhara K.** 2005. Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. Nature **437:**898–901. https://doi.org/10.1038/nature04033.
42. **Kiyokawa Y, Kikusui T, Takeuchi Y, Mori Y.** 2006. Removal of the vomeronasal organ blocks the stress-induced hyperthermia response to alarm pheromone in male rats. Chem Senses **32:**57–64. https://doi.org/10.1093/chemse/bjl036.
43. **Lacey JC, Beynon RJ, Hurst JL.** 2007. The importance of exposure to other male scents in determining competitive behaviour among inbred male mice. Appl Anim Behav Sci **104:**130–142. https://doi.org/10.1016/j.applanim.2006.04.026.
44. **Lateef DM, Abreu-Vieira G, Xiao C, Reitman ML.** 2014. Regulation of body temperature and brown adipose tissue thermogenesis by bombesin receptor subtype-3. Am J Physiol Endocrinol Metab **306:**E681–E687. https://doi.org/10.1152/ajpendo.00615.2013.
45. **Latham N, Mason G.** 2004. From house mouse to mouse house: the behavioural biology of free-living *Mus musculus* and its implications in the laboratory. Appl Anim Behav Sci **86:**261–289. https://doi.org/10.1016/j.applanim.2004.02.006.
46. **Lee DL, Webb RC, Brands MW.** 2004. Sympathetic and angiotensin-dependent hypertension during cage-switch stress in mice. Am J Physiol Regul Integr Comp Physiol **287:**R1394–R1398. https://doi.org/10.1152/ajpregu.00306.2004.
47. **Lee DL, Leite R, Fleming C, Pollock JS, Webb RC, Brands MW.** 2004. Hypertensive response to acute stress is attenuated in interleukin-6 knockout mice. Hypertension **44:**259–263. https://doi.org/10.1161/01.HYP.0000139913.56461.fb.
48. **Lipman NS, Corning BF, Coiro MA Sr.** 1992. The effects of intracage ventilation on microenvironmental conditions in filter-top cages. Lab Anim **26:**206–210. https://doi.org/10.1258/002367792780740503.
49. **Lipman NS, Homberger FR.** 2003. Rodent quality assurance testing: use of sentinel animal systems. Lab Anim **32:**36–43. https://doi.org/10.1038/laban0503-36.
50. **Mackintosh JH, Grant EC.** 1966. The effect of olfactory stimuli on the agonistic behaviour of laboratory mice. Z Tierpsychol **23:**584–587. https://doi.org/10.1111/j.1439-0310.1966.tb01614.x.
51. **Mähler Convenor M, Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning K, Raspa M.** 2014. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. Lab Anim (NY) **48:**178–192. https://doi.org/10.1177/0023677213516312.
52. **Manzano Nieves G, Schilit Nitenson A, Lee HI, Gallo M, Aguilar Z, Johnsen A, Bravo M, Bath KG.** 2019. Early Life Stress Delays Sexual Maturation in Female Mice. Front Mol Neurosci **12:**1–12. https://doi.org/10.3389/fnmol.2019.00027.
53. **McEwen BS, Milner TA.** 2016. Understanding the broad influence of sex hormones and sex differences in the brain. J Neurosci Res **95:**24–39. https://doi.org/10.1002/jnr.23809.
54. **Meaney MJ, Diorio J, Francis D, Widdowson J, LaPlante P, Caldji C, Sharma S, Seckl JR, Plotsky PM.** 1996. Early environmental regulation of forebrain glucocorticoid receptor gene expression: Implications for adrenocortical responses to stress. Dev Neurosci **18:**49–60. https://doi.org/10.1159/000111395.
55. **Mexas AM, Brice AK, Caro AC, Hillanbrand TS, Gaertner DJ.** 2015. Nasal histopathology and intracage ammonia levels in female groups and breeding mice housed in static isolation cages. J Am Assoc Lab Anim Sci **54:**478–486.
56. **Miller DB, O'Callaghan JB.** 2002. Neuroendocrine aspects of the response to stress. Metabolism **51 Suppl 1:**5–10. https://doi.org/10.1053/meta.2002.33184.
57. **Mohrhardt J, Nagel M, Fleck D, Ben-Shaul Y, Spehr M.** 2018. Signal detection and coding in the accessory olfactory system. Chem Senses **43:**667–695. https://doi.org/10.1093/chemse/bjy061.
58. **Moncho-Bogani J, Lanuza E, Hernández A, Novejarque A, Martínez-García F.** 2002. Attractive properties of sexual pheromones in mice. Physiol Behav **77:**167–176. https://doi.org/10.1016/S0031-9384(02)00842-9.
59. **Montani G, Tonelli S, Sanghez V, Ferrari PF, Palanza P, Zimmer A, Tirindelli R.** 2013. Aggressive behaviour and physiological responses to pheromones are strongly impaired in mice deficient for the olfactory G-protein -subunit Gy8. J Physiol **591:**3949–3962. https://doi.org/10.1113/jphysiol.2012.247528.
60. **Morton DB, Griffiths PHM.** 1985. Guidelines on the recognition of pain, distress, and discomfort in experimental animals and an hypothesis for assessment. Vet Rec **116:**431–436. https://doi.org/10.1136/vr.116.16.431.
61. **Muscogiuri G, Cantone E, Cassarano S, Tuccinardi D, Barrea L, Savastano S, Colao A.** 2019. Gut microbiota: a new path to treat obesity. Int J Obes Suppl **9:**10–19. https://doi.org/10.1038/s41367-019-0011-7.
62. **National Research Council (US) Committee on Recognition and Alleviation of Distress in Laboratory Animals.** 2008. Stress and distress: definitions, p. 13-24. In: Recognition and alleviation of distress in laboratory animals. Washington (DC): National Academies Press.
63. **Novak J, Bailoo JD, Melotti L, Rommen J, Würbel H.** 2015. An exploration based cognitive bias test for mice: effects of handling method and stereotypic behaviour. PLoS One **10:**e0130718. https://doi.org/10.1371/journal.pone.0130718.
64. **Novak J, Bailoo JD, Melotti L, Würbel H.** 2016. Effect of cage-induced stereotypies on measures of affective state and recurrent perseveration in CD-1 and C57BL/6 mice. PLoS One **11:**e0153203. https://doi.org/10.1371/journal.pone.0153203.
65. **Novotny M, Harvey S, Jemiolo B, Alberts J.** 1985. Synthetic pheromones that promote inter-male aggression in mice. Proc Natl Acad Sci USA **82:**2059–2061. https://doi.org/10.1073/pnas.82.7.2059.
66. **O'Leary TP, Gunn RK, Brown RE.** 2013. What are we measuring when we test strain differences in anxiety in mice? Behav Genet **43:**34–50. https://doi.org/10.1007/s10519-012-9572-8.
67. **Palanza P.** 2001. Animal models of anxiety and depression: how are females different? Neurosci Biobehav Rev **25:**219–233. https://doi.org/10.1016/S0149-7634(01)00010-0.
68. **Pallas BD, Keys DM, Bradley MP, Vernasco-Price EJ, Sanders JD, Allen PS, Freeman ZT.** 2020. Compressed paper as an alternative

to corn cob bedding in mouse (*Mus musculus*) cages. J Am Assoc Lab Anim Sci **59:**496–502. https://doi.org/10.30802/AALAS-JAALAS-19-000151.
69. **Potgieter FJ, Wilke PI.** 1996. The dust content, dust generation, ammonia production, and absorption properties of three different rodent bedding types. Lab Anim **30:**79–87. https://doi.org/10.1258/002367796780744893.
70. **Razzoli M, Bartolomucci A.** 2016. The dichotomous effect of chronic stress on obesity. Trends Endocrinol Metab **27:**504–515. https://doi.org/10.1016/j.tem.2016.04.007.
71. **Razzoli M, Cero C, Bartolomucci A.** 2017. Chapter 4: How does stress affect energy balance? p. 53-89. In: Tucci B, editor. Handbook of neurobehavioral genetics and phenotyping. Hoboken (NJ): John Wiley & Sons.
72. **Roberts SA, Simpson DM, Armstrong SD, Davidson AJ, Robertson DH, McLean L, Beynon RJ, Hurst JL.** 2010. Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. BMC Biol **8:**75. https://doi.org/10.1186/1741-7007-8-75.
73. **Rock ML, Karas AZ, Rodriguez KB, Gallo MS, Pritchett-Corning K, Karas RH, Aronovitz M, Gaskill BN.** 2014. The time-to-integrate-to-nest test as an indicator of wellbeing in laboratory mice. J Am Assoc Lab Anim Sci **53:**24–28.
74. **Ropartz P.** 1968. The relation between olfactory stimulation and aggressive behaviour in mice. Anim Behav **16:**97–100. https://doi.org/10.1016/0003-3472(68)90117-6.
75. **Rosenbaum MD, VandeWoude S, Johnson TE.** 2009. Effects of cage-change frequency and bedding volume on mice and their microenvironment. J Am Assoc Lab Anim Sci **48:**763–773.
76. **Rottman SJ, Snowdon CT.** 1972. Demonstration and analysis of an alarm pheromone in mice. J Comp Physiol Psychol **81:**483–490. https://doi.org/10.1037/h0033703.
77. **Sachs BD, Ni JR, Caron MG.** 2014. Sex differences in response to chronic mild stress and congenital serotonin deficiency. Psychoneuroendocrinology **40:**123–129. https://doi.org/10.1016/j.psyneuen.2013.11.008.
78. **Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, Hammer RE, Williams SC, Crowley J, Yanagisawa M.** 2008. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci USA **105:**16767–16772. https://doi.org/10.1073/pnas.0808567105.
79. **Schmidt MV, Scharf SH, Liebl C, Harbich D, Mayer B, Holsboer F, Müller MB.** 2010. A novel chronic social stress paradigm in female mice. Horm Behav **57:**415–420. https://doi.org/10.1016/j.yhbeh.2010.01.010.
80. **Spencer RL, Deak T.** 2017. A users guide to HPA axis research. Physiol Behav **178:**43–65. https://doi.org/10.1016/j.physbeh.2016.11.014.
81. **Steimer T.** 2011. Animal models of anxiety disorders in rats and mice: some conceptual issues. Dialogues Clin Neurosci **13:**495–506. https://doi.org/10.31887/DCNS.2011.13.4/tsteimer.
82. **Tataryn NM, Buckmaster CA, Schwiebert RS, Swennes AG.** 2021. Comparison of four beddings for ammonia control in individually ventilated mouse cages. J Am Assoc Lab Anim Sci **60:**37–43. https://doi.org/10.30802/AALAS-JAALAS-20-000051.
83. **ter Horst JP, de Kloet ER, Schächinger H, Oitzl MS.** 2011. Relevance of stress and female sex hormones for emotion and cognition. Cell Mol Neurobiol **32:**725–735. https://doi.org/10.1007/s10571-011-9774-2.
84. **Tournier JN, Mathieu J, Mailfert Y, Multon E, Drouet C, Jouan A, Drouet E.** 2001. Chronic restraint stress induces severe disruption of the T-cell specific response to tetanus toxin vaccine. Immunology **102:**87–93. https://doi.org/10.1046/j.1365-2567.2001.01152.x.
85. **Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI.** 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature **444:**1027–1031. https://doi.org/10.1038/nature05414.
86. **Valsamakis G, Chrousos G, Mastorakos G.** 2019. Stress, female reproduction and pregnancy. Psychoneuroendocrinology **100:**48–57. https://doi.org/10.1016/j.psyneuen.2018.09.031.
87. **Vandenbergh JG.** 1967. Effect of the presence of a male on the sexual maturation of female mice. Endocrinology **81:**345–349. https://doi.org/10.1210/endo-81-2-345.
88. **Van Der Lee S, Boot LM.** 1955. Spontaneous pseudopregnancy in mice. Acta Physiol Pharmacol Neerl **4:**442–444.
89. **van Doeselaar L, Yang H, Bordes J, Brix L, Engelhardt C, Tang F, Schmidt MV.** 2021. Chronic social defeat stress in female mice leads to sex-specific behavioral and neuroendocrine effects. Stress **2:**168–180.
90. **Van Oortmerssen GA.** 1971. Biological significance, genetics and evolutionary origin of variability in behaviour within and between inbred strains of mice (*Mus musculus*): a behaviour genetic study. Behaviour **38:**1–91. https://doi.org/10.1163/156853971X00014.
91. **Vogelweid CM, Zapien KA, Honigford MJ, Li L, Li H, Marshall H.** 2011. Effects of a 28-day cage-change interval on intracage ammonia levels, nasal histology, and perceived welfare of CD1 mice. J Am Assoc Lab Anim Sci **50:**868–878.
92. **Whitten WK.** 1956. Modification of the oestrous cycle of the mouse by external stimuli associated with the male. J Endocrinol **13:**399–404. https://doi.org/10.1677/joe.0.0130399.
93. **Würbel H, Stauffacher M.** 1997. Age and weight at weaning affect corticosterone level and development of stereotypies in ICR-mice. Anim Behav **53:**891–900. https://doi.org/10.1006/anbe.1996.0424.
94. **Würbel H, Stauffacher M, von Holst D.** 1996. Stereotypies in laboratory mice — quantitative and qualitative description of the ontogeny of 'wire-gnawing' and 'jumping' in Zur:ICR and Zur:ICR nu. Ethology **102:**371–385. https://doi.org/10.1111/j.1439-0310.1996.tb01133.x.
95. **Yokosuka M, Matsuoka M, Ohtani-Kaneko R, Iigo M, Hara M, Hirata K, Ichikawa M.** 1999. Female-soiled bedding induced fos immunoreactivity in the ventral part of the premammillary nucleus (PMv) of the male mouse. Physiol Behav **68:**257–261. https://doi.org/10.1016/S0031-9384(99)00160-2.