

# Evaluation of Cage Micro-Environment of Mice Housed on Various Types of Bedding Materials

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A variety of environmental factors can affect the outcomes of studies using laboratory rodents. One such factor is bedding. Several new bedding materials and processing methods have been introduced to the market in recent years, but there are few reports of their performance. In the studies reported here, we have assessed the cage micro-environment (in-cage ammonia levels, temperature, and humidity) of mice housed on various kinds of bedding and their combinations. We also compared results for bedding supplied as Nestpaks versus loose bedding. We studied C57BL/6J mice (commonly used) and NOD/LtJ mice (heavy soilers) that were maintained, except in one study, in static duplex cages. In general, we observed little effect of bedding type on in-cage temperature or humidity; however, there was considerable variation in ammonia concentrations. The lowest ammonia concentrations occurred in cages housing mice on hardwood bedding or a mixture of corncob and alpha cellulose. In one experiment comparing the micro-environments of NOD/LtJ male mice housed on woodpulp fiber bedding in static versus ventilated caging, we showed a statistically significant decrease in ammonia concentrations in ventilated cages. Therefore, our data show that bedding type affects the micro-environment in static cages and that effects may differ for ventilated cages, which are being used in vivaria with increasing frequency.

Outcomes of studies with laboratory animals can depend heavily on multiple environmental factors. In the case of rodents, one such factor is the type of bedding. Physiologic changes may occur after exposure to some types of bedding and could affect experimental results. Some bedding generates dust and particulates that might cause respiratory or ocular changes. Bedding that is very absorbent could reduce operating costs.

In 1980, Kraft (1) published a review of bedding available at that time for laboratory rodents. She listed several desirable characteristics of bedding—among other characteristics, it should be moisture absorbent, inedible, non-traumatic, nontoxic, readily available, relatively inexpensive, non-deleterious to cage washers, and free of dust and splinters. At the time the article was published, white pine shavings were the most commonly used rodent bedding. It was known at that time, however, that cedar and white pine shavings should not be used as bedding for rodents used in pharmacologic studies. Some other rather exotic bedding had been assessed: hay (edible) and peat moss, newsprint, and alfalfa (stained animals' coats). Pelleted peanut hulls had recently been introduced to the market but had not been fully evaluated. Their absence from today's market suggests that they were not satisfactory. Natural products are prone to variability and, perhaps, microbial or chemical contamination.

Dr. Kraft ended her review with the following questions: "Taking into account scientific, economic, humane, and legal aspects of laboratory animal bedding, is there agreement that there should be standards for bedding? And who will do the work in order to obtain the results on which the standards are to be based?" (1). Recently available products such as cellulose, corn cob, recycled paper, and Nestpak bedding are gaining popularity but may not have been fully evaluated. For example, variations in absorbency can affect both in-cage humidity and the microflora that convert urea into ammonia. Here we report an assessment of the environment—temperature, relative humidity, and ammonia concentrations—within cages housing NOD/LtJ or C57BL/6J mice on several bedding types or combinations thereof.

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## Materials and Methods

**Mice.** We obtained eight-week-old NOD/LtJ (NOD) and C57BL/6J (B6) male mice as well as pairs of B6 breeder mice from JAX Research Systems (JRS; Bar Harbor, Maine). All JRS colonies are regularly monitored for and are free of 15 viruses (mouse hepatitis virus, two mouse parvoviruses, reovirus, Theiler's mouse encephalomyelitis virus, ectromelia virus, mouse rotavirus, thymic virus, pneumonia virus of mice, Sendai virus, murine cytomegalovirus, lactic dehydrogenase-elevating virus, K virus, mouse adenovirus, and polyoma virus), 17 bacterial species (including *Helicobacter* spp.), two *Mycoplasma* spp., external and intestinal parasites, and *Encephalitozoon cuniculi*. In addition, fecal samples from the mice were tested for *Proteus* species prior to study and were negative. Mice were housed in static polycarbonate duplex cages (floor space, 51.7 in<sup>2</sup>) with loose-fitting Reemay filters (Reemay 2033, Thoren Caging Systems, Inc., Hazleton, Pa.). One study compared ammonia concentrations in static versus positively ventilated cages at an anemometer reading of 0.025 Pascals (0.0001 in. of water). The duplex cage (Thoren Caging Systems, Inc.) is divided into two pens with wire-rod tops to hold the water bottles and diet for each pen. In each duplex cage, one side held four B6 or NOD male mice or a litter of B6 mice with both parents. The mice were provided ad libitum with acidified water (pH 2.8 to 3.1, monitored continuously) and pelleted 5K52 (modified from the NIH 31M open formula; 6% fat) diet (PMI Nutrition International, Brentwood, Mo.) that was autoclaved at 100°C for 58 min. The room in which the mice were housed was supplied with HEPA-filtered air at 19 air changes per hour and was maintained at a temperature (mean  $\pm$  standard error) of 22  $\pm$  2°C, relative humidity of 35%  $\pm$  4%, and a 14:10-h light:dark cycle. Bedding was autoclaved and changed after 3 weeks, except where noted in the Results. Manipulation of cages occurred in a Maxi-Miser (Thoren Caging Systems, Inc.) mobile ventilated cage-changing station. The bedding types, sources, manufacturers and/or distributors, and amounts used per cage are given in Table 1. Except for pine shavings, the bedding amounts were based on manufacturers' recommendations.

**Micro-environmental monitoring.** For each experiment described below, in-cage temperature, relative humidity (RH), and ammonia concentrations were measured using an INNOVA multi-gas analyzer

Table 1. Types and sources of bedding and amounts used per cage

Bedding name	Bedding material	Source	Amount per cage (in grams or depth in inches)
ALPHA-dri	Alpha cellulose	Shepherd's, Watertown, Tenn.	3/8"
Bed-O'cobs	Corncob	The Andersons, Maumee, Ohio	1/4"
Beta Chip	Hardwood (maple, beech, poplar)	Northeastern Products Corp., Warrensburg, N.Y.	5/8"
Cell-Sorb Plus	Recycled newspaper	Fangman Specialties, Inc., Cincinnati, Ohio	100 g (7-day change) 150 g (14-day change) 200 g (21-day change)
CareFRESH Ultra	Long-fiber, high-grade bleached pulp	Absorption Corp., Bellingham, Wash.	1"
Shavings	Pine	Crobb Box, Ellsworth, Maine	5/8" (The Jackson Laboratory standard)
Nestpaks	Bed-O'cobs ALPHA-dri ALPHA-dri plus Bed-O'cobs Beta chip	WF Fisher and Son Inc., Somerville, N.J.	Manufacturer's recommendations

(Innova AirTech Instruments A/S, Ballerup, Denmark). The details of the monitoring have been described by Reeb et al. (2). Briefly, cages used for monitoring had small ports drilled in their walls, and metal fittings, which could be sealed when not in use, were attached. The gas analyzer probes were inserted through the ports for monitoring. Measurements, three for each cage on each measurement day, were made on Monday, Wednesday, and Friday for 3 weeks during each experiment. If the ammonia levels exceeded 200 ppm within a cage, the mice in that cage were housed on clean bedding, and the micro-environmental measurements were discontinued.

**The 3-week experiment with male NOD/LtJ mice.** Data were collected over various times of the calendar year, so season was included as a treatment effect. Winter included dates from December through February; spring, March through May; and summer, June through August. No data were collected in autumn. An analysis of covariance (ANCOVA) model was used to test for differences in micro-environmental variables among bedding types and season, with time (day of the experiment) as a covariate. Ammonia data were  $\log_e$ -transformed to stabilize variances. Tukey's honestly significant differences (HSD) was used for multiple comparisons when significant differences ( $\alpha = 0.05$ ) were found. All analyses were performed using JMP [Version 5.0.1.2, The SAS Institute, Inc., Cary, N.C.].

**The 2-week experiment with male NOD/LtJ mice.** In light of results from the preceding experiment, we conducted a second experiment to compare micro-environmental measures in static cages with CareFRESH Ultra bedding or pine shavings. Mice were housed for 2 weeks on one or the other bedding (three cages for each bedding type). An ANCOVA model was used to test for differences between bedding (main treatment), time (covariate), and their interaction.

**The 3-week experiment with male C57BL/6J mice housed on loose versus Nestpak bedding.** We used a crossover design to test for differences in environmental measures between cages with loose and Nestpak bedding. Male B6 mice were housed for 3 weeks on four types of loose or corresponding Nestpak bedding. Mice that spent their first 3-week rotation on loose bedding were switched after 3 weeks to the corresponding Nestpak bedding and vice versa. Each bedding type was tested on 6 cages of mice, and we collected 54 measurements for each rotation. We statistically compared a single bedding type supplied as either loose bedding or Nestpaks (we did not compare among different types of loose bedding or different types of Nestpaks). We tested for sequence, period (time of year), and treatment effects, using  $\alpha = 0.05$ .

**The 3-week experiment with C57BL/6J breeder pairs with offspring.** This experiment was designed to determine micro-environmental differences for cages housing breeding pairs of B6 mice housed with their

offspring for 21 days post-partum. Breeder pairs were randomly assigned to 6 cages per bedding type. Litter sizes varied from 1 to 10, with an average litter size of 5.7 housed on pine bedding, 5.0 on Care Fresh, 4.8 on Bed-O'cobs, 6.0 on Cell-Sorb Plus, 5.2 on Beta Chip, 5.0 on Bed-O'cobs plus ALPHA-dri, and 7.3 on pine plus ALPHA-dri. Data were analyzed using an ANCOVA model with bedding as the treatment and time and number of pups as covariates. Ammonia data were  $\log_e$ -transformed to stabilize variances, and we used  $\alpha = 0.05$ .

**Microscopic evaluation of nasal passages.** To evaluate possible ammonia toxicity, four mice from each of four groups were euthanized with carbon dioxide gas at the end of each study. The groups were selected from cages that had < 25, 25 to 50, 50 to 100, or > 100 ppm of ammonia at the end of three weeks. The noses were collected, fixed for 24 h in Bouin's fixative, and washed multiple times in running water. The tissues were processed through alcohols and xylene and then embedded in Paraplast tissue embedding medium (Pelco International, Redding, Calif.) for sectioning at 5  $\mu$ m followed by staining with hematoxylin and eosin. We examined the nose of each mouse at five different levels.

The Institutional Animal Care and Use Committee approved all of the reported experiments, including a variance from the normal cage-changing interval and a reduction in floor space per mouse relative to recommendations in the *Guide to the Care and Use of Laboratory Animals*. Housing four B6 or NOD mice per cage resulted in approximately 12.9 in<sup>2</sup> floor space per mouse.

## Results

**The 3-week experiment with male NOD/LtJ mice.** Differences in temperature were not systematically related to bedding material and varied only 1.1°C (24.0°C to 25.1°C) between the lowest and highest averages for the three seasons. Least-squares means ( $\pm$  standard error) of RH for all control cages (with bedding but no mice) were significantly lower (39.5%  $\pm$  0.1%) than those for cages housing mice (60.8%  $\pm$  2.4%). The mean RH in cages housing mice on the Bed-O'cobs plus ALPHA-dri (56.6%  $\pm$  0.7%) was significantly lower than that for all other tested bedding material except CareFRESH Ultra (59.1%  $\pm$  2.0%). All RH values were within the range (30% to 70%) recommended in the *Guide*.

The mean ammonia concentrations in static cages housing mice on CareFRESH Ultra bedding were significantly higher than the means for all the other bedding treatments (Table 2). Unexpectedly, the ammonia concentrations in cages housing mice on CareFRESH Ultra bedding were very high at 2 weeks (65.4 to 390.2 ppm), so we removed that group from the study. Mean ammonia levels in cages

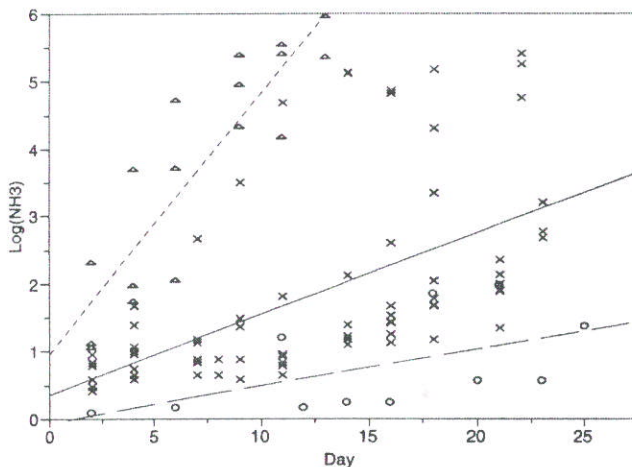
**Table 2.** Least squares means ammonia concentrations in static cages housing male NOD/LtJ mice on different types of bedding for 3 weeks

Bedding type	Ammonia concentration (ppm; mean $\pm$ standard error)
Care FRESH Ultra <sup>a</sup>	122.7 $\pm$ 1.5 <sup>A</sup>
Pine shavings	13.2 $\pm$ 1.1 <sup>B</sup>
Cell-Sorb Plus	10.6 $\pm$ 1.2 <sup>B</sup>
Pine shavings plus ALPHA-dri	10.5 $\pm$ 1.2 <sup>B</sup>
Bed-O'cobs	8.6 $\pm$ 1.1 <sup>B</sup>
Beta Chip	3.8 $\pm$ 1.2 <sup>C</sup>
Bed-O'cobs and ALPHA-dri	2.5 $\pm$ 1.2 <sup>C,D</sup>
Room	1.9 $\pm$ 1.2 <sup>C,D</sup>
Pine shavings control <sup>b</sup>	1.4 $\pm$ 1.3 <sup>C,D</sup>
Beta Chip control <sup>b</sup>	1.2 $\pm$ 1.3 <sup>D</sup>
Bed-O'cobs and ALPHA-dri control <sup>b</sup>	1.2 $\pm$ 1.2 <sup>D</sup>

Values not labeled with the same letter are statistically different from one another, based on Tukey HSD test with  $\alpha = 0.05$ .

<sup>a</sup>Discontinued after 2 weeks.

<sup>b</sup>Control boxes contained bedding but no mice.



**Figure 1.** Log<sub>10</sub>-transformed ammonia levels for three bedding materials as a function of time. Mice were adult NOD/LtJ males that were housed 4 per cage for 2 weeks on CareFRESH Ultra bedding (triangles and dotted line) or 3 weeks on Bed-O'cobs (X's and solid line). The circles and dashed line indicate ammonia concentrations in cages with pine bedding but no mice.

with pine shavings, Cell-Sorb Plus, a mixture of pine shavings and ALPHA-dri, and Bed-O'cobs did not differ significantly from each other but were significantly different from other bedding types (Table 2). Ammonia levels in cages housing mice on Bed-O'cobs mixed with ALPHA-dri (2.5 ppm) were the lowest and did not differ significantly from those of all of the controls (cages with bedding but no mice). Figure 1 shows the increase over time of ammonia concentrations in cages housing NOD male mice on CareFRESH versus Bed-O'cobs.

**The 2-week experiment with male NOD/LtJ mice.** To confirm the unexpected results for CareFRESH Ultra bedding, we performed a second 2-week study to compare ammonia concentrations in cages of male NOD mice housed on pine shavings or CareFRESH Ultra (Table 3). The least squares means ammonia concentrations in cages with CareFRESH Ultra bedding (44.7  $\pm$  1.2 ppm) were lower than those in the prior experiment but significantly higher than in cages with pine bedding (11.2  $\pm$  1.2 ppm). There was no significant effect of bedding, time, or their interaction for both temperature and RH.

**The 3-week experiment with C57BL/6J male mice housed on loose versus Nestpak bedding.** Several bedding types are supplied either as loose bedding or as Nestpaks, which resemble large tea bags

**Table 3.** Least squares means ammonia concentrations in static cages housing male NOD/LtJ mice on CareFRESH Ultra or pine shavings for 2 weeks

Bedding type	Ammonia concentration (ppm, mean $\pm$ standard error)
Care FRESH Ultra	44.7 $\pm$ 1.2 <sup>A</sup>
Pine shavings	11.2 $\pm$ 1.2 <sup>B</sup>

Values not connected by the same letter are significantly different from each other, based on Tukey HSD test with  $\alpha = 0.05$ .

**Table 4.** Least squares means ammonia concentrations in static cages housing C57BL/6J breeder pairs and litters on different types of bedding for 3 weeks

Bedding type	Mean ammonia concentration (ppm, mean $\pm$ standard error)
CareFRESH Ultra	30.0 $\pm$ 1.2 <sup>A</sup>
Pine Shavings	10.1 $\pm$ 1.1 <sup>B</sup>
Pine Shavings plus ALPHA-dri	6.3 $\pm$ 1.2 <sup>B,C</sup>
Cell-Sorb Plus	5.0 $\pm$ 1.1 <sup>C</sup>
Bed-O'cobs	4.7 $\pm$ 1.1 <sup>C</sup>
Beta Chip	4.1 $\pm$ 1.1 <sup>C,D</sup>
Bed-O'cobs plus ALPHA-dri	2.6 $\pm$ 1.1 <sup>D</sup>

Values not labeled with the same letter are significantly different from each other based on Tukey HSD test with  $\alpha = 0.05$ .

and are frequently used to provide environmental enrichment for mice. The treatment effect (Nestpak versus loose) was not significant for any bedding type; that is, the ammonia levels in cages with loose bedding did not differ from those with Nestpaks containing the same bedding. There was no noted sequence effect on ammonia concentration. However, there was a significant period effect: in every case, least squares means ammonia values were significantly higher ( $P < 0.0001$ ) in January than in December. The in-cage temperatures were also higher in January than in December. The treatment effect was significant only for Bed-O'cobs mixed with ALPHA-dri ( $P = 0.04$ ), although the difference in least square mean estimates was only 0.3°C (loose, 24.7°C; Nestpak, 24.4°C). For RH, the overall ANOVA model was significant only for ALPHA-dri ( $P = 0.01$ ), although the results of none of the effects tests (sequence, period, treatment) were significant for this bedding type.

**The 3-week experiment with C57BL/6J breeder pairs with offspring.** After adjusting for the covariates day and number of pups, the mean ammonia values with CareFRESH Ultra bedding had a significantly higher least squares mean (30.0  $\pm$  1.2 ppm) than did all the other bedding treatments (Table 4). Ammonia concentrations in cages housing mice on pine shavings or a mixture of pine shavings plus ALPHA-dri were not significantly different from each other, although pine shavings yielded significantly higher mean ammonia levels (10.1  $\pm$  1.1 ppm) than did all of the remaining bedding types. Bed-O'cobs mixed with ALPHA-dri yielded significantly lower ammonia levels than all other bedding types except Beta Chip. For all bedding types tested except CareFRESH Ultra, ammonia concentrations remained at or below 20 ppm. Ammonia concentrations in cages of mice housed on CareFRESH Ultra bedding were 20 ppm at week 1 and escalated to 120 ppm by 3 weeks.

Temperatures (range, 23.5°C to 25.7°C) did not differ significantly among the bedding types except for cages housing mice on corn cob, which had significantly lower temperatures than the other bedding types. RH values (range, 48% to 62%) were unaffected by bedding type.

**The 3-week experiment with NOD/LtJ male mice housed on two bedding types in static or ventilated caging.** Ammonia levels