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

ELLEN SMITH, JASON D. STOCKWELL, PHD,[†] ISABELLE SCHWEITZER, MS, STEPHEN H. LANGLEY, BS, AND ABIGAIL L. SMITH, MPH, PHD[*]

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.

The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609

[†]Present address: USGS-Great Lakes Science Center, Lake Superior Biological Station, 2800 Lakeshore Dr., Ashland, Wisconsin 54806

[*]Corresponding author

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^2) 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 ± standard error) of 22 ± 2°C, relative humidity of 35% ± 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 μm 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^2 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 (± standard error) of RH for all control cages (with bedding but no mice) were significantly lower (39.5% ± 0.1%) than those for cages housing mice (60.8% ± 2.4%;). The mean RH in cages housing mice on the Bed-O'cobs plus ALPHA-dri (56.6% ± 0.7%) was significantly lower than that for all other tested bedding material except CareFRESH Ultra (59.1% ± 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 ± standard error)
Care FRESH Ultra[a]	122.7 ± 1.5[A]
Pine shavings	13.2 ± 1.1[B]
Cell-Sorb Plus	10.6 ± 1.2[B]
Pine shavings plus ALPHA-dri	10.5 ± 1.2[B]
Bed-O'cobs	8.6 + 1.1[B]
Beta Chip	3.8 ± 1.2[C]
Bed-O'cobs and ALPHA-dri	2.5 ± 1.2[C,D]
Room	1.9 ± 1.2[C,D]
Pine shavings control[b]	1.4 ± 1.3[C,D]
Beta Chip control[b]	1.2 ± 1.3[D]
Bed-O'cobs and ALPHA-dri control[b]	1.2 ± 1.2[D]

Values not labeled with the same letter are statistically different from one another, based on Tukey HSD test with $\alpha = 0.05$.
[a]Discontinued after 2 weeks.
[b]Control boxes contained bedding but no mice.

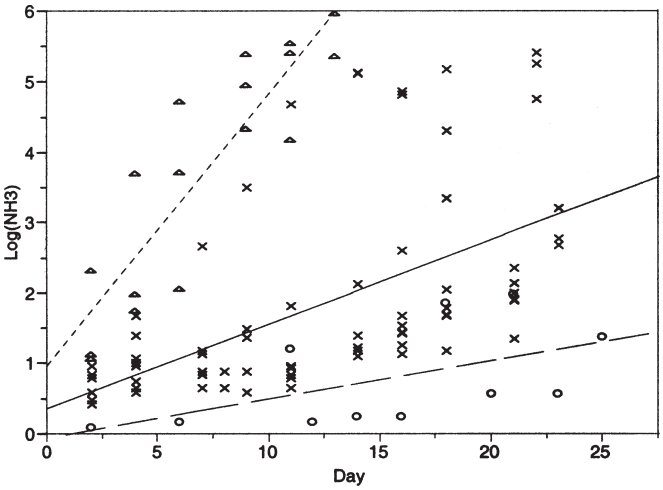

Figure 1. Log_e-transformed ammonia levels for three bedding materials as a function of time. Mice were adult NOD/LtJ males that were housed 4 per cage for 2 weeks on CareFRESH Ultra bedding (triangles and dotted line) or 3 weeks on Bed-O'cobs (X's and solid line). The circles and dashed line indicate 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 ± 1.2 ppm) were lower than those in the prior experiment but significantly higher than in cages with pine bedding (11.2 ± 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 ± standard error)
Care FRESH Ultra	44.7 ± 1.2[A]
Pine shavings	11.2 ± 1.2[B]

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 ± standard error)
CareFRESH Ultra	30.0 ± 1.2[A]
Pine Shavings	10.1 ± 1.1[B]
Pine Shavings plus ALPHA-dri	6.3 ± 1.2[B,C]
Cell-Sorb Plus	5.0 ± 1.1[C]
Bed-O'cobs	4.7 ± 1.1[C]
Beta Chip	4.1 ± 1.1[C,D]
Bed-O'cobs plus ALPHA-dri	2.6 ± 1.1[D]

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 ± 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 ± 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

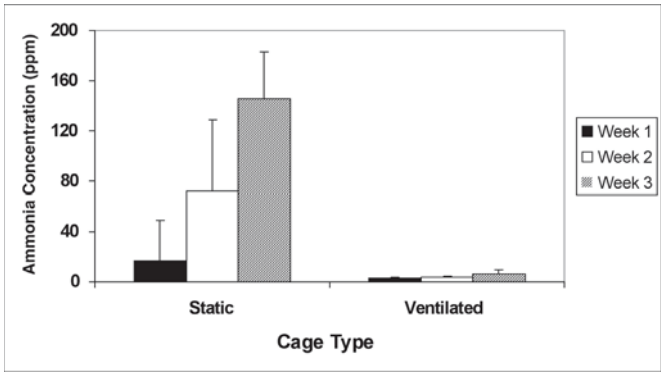

Figure 2. Comparison of least squares means ammonia concentrations in static and ventilated cages housing adult NOD/LtJ male mice 4 per cage on CareFRESH Ultra bedding for 3 weeks.

in static cages housing male NOD mice and B6 breeder pairs with offspring on CareFRESH Ultra bedding were significantly higher ($P < 0.0001$) than those for the other bedding types. We reasoned that this bedding might not dry out when saturated in static cages and, therefore, we did a comparative study using NOD male mice housed on CareFRESH Ultra bedding in static and ventilated caging systems. As shown in Fig. 2, ammonia levels in static cages were substantially higher than those in ventilated cages. At 2 and 3 weeks, ammonia levels in static cages were > 20-fold higher than those in ventilated cages. One CareFRESH Ultra–ventilated cage data point from the third week was omitted from the analysis: the single point was 143.4 ppm, whereas the range without that information was 3.4 to 16.1 ppm. The mean ammonia concentrations in the static cages containing CareFRESH Ultra bedding reached 145.6 ppm by the third week of the study.

Nasal passages from selected mice in these studies were histologically normal irrespective of in-cage ammonia levels.

Discussion

A few published studies have compared different types of bedding for housing mice and other rodents. A very early report indicated that compared with mice housed on pine sawdust, SCH:ARSHa (ICR) mice housed on corncob bedding had reduced reproductive success, with a 10% drop in the number of mice weaned (3). In addition, 21 of 24 mice housed on sawdust bore young compared with 16 of 24 housed on corncob bedding, although these differences are not statistically significant. Several explanations were offered, including the possible presence of a mycotoxin in the corncob bedding, but the reason was not definitively identified.

Cotton bedding has been associated with conjunctivitis in athymic nude mice (4). Another report compared mucosal immune responses of mice housed on cotton or wood bedding. Mice housed on wood bedding had increased numbers of Peyer's patches. Cultured Peyer's patch and mesenteric lymph node lymphocytes from mice housed on wood also produced higher levels of total and virus-specific IgA (5). One could speculate that some contaminant in the wood bedding provided chronic stimulation of the gastrointestinal immune system. Serum virus-specific antibody responses were unaffected (5).

Several studies have compared the microsomal enzymes in livers of mice housed on different types of bedding. One early study (6) described the results for 10 inbred and 2 outbred strains of mice as well as Sprague-Dawley rats. Compared with animals housed on hardwood (mixed beech, birch, and maple) shavings, those housed on softwood bedding (red cedar, white pine, or ponderosa pine) had decreased sleep times and increased liver microsomal enzymes that metabolize hexobarbital. Another study concluded that even the low levels of aromatic hydrocarbons that may be present in cedar, eucalyptol in aerosol sprays, and chlorinated hydrocarbon insecticides can increase microsomal enzyme activity in rodent livers, whereas ammonia generated from feces and urine that accumulate in cages can inhibit enzyme activity (7). In another study, red cedar shavings used as bedding increased liver microsomal enzymes, reduced barbiturate sleep times, increased the incidence of spontaneous liver and mammary gland tumors in susceptible mouse strains, and reduced the average time at which the tumors occurred (8). More recently, Cunliffe-Beamer and colleagues demonstrated that, compared to non-autoclaved bedding, autoclaving mixed hardwood, white spruce, white pine, or red cedar shavings did not alter barbiturate sleep times or liver:body weight ratios of DBA/2J and C57BL/6J male mice (9). However, the sleep times of mice on mixed hardwood or white spruce bedding were significantly longer than those of mice on white pine or red cedar bedding. Liver:body weight ratios in both strains housed on red cedar bedding were significantly elevated compared with those of mice housed on white pine, white spruce, or mixed hardwood beddings (9). A very recent report has shown that removal of rats from pine bedding to wire-bottomed cages results in variable rates of enzyme activity decline (10), and 6 to 12 weeks were required for enzyme activity to stabilize. Taken together, these studies indicate that the bedding on which mice are housed may influence pharmacologic responsiveness of mice to a variety of drugs and may explain discrepancies in the drug metabolism literature.

Corning and Lipman (11) studied micro-environments of male and female DBA/2J and Crl:CD-1(ICR)BR mice with conventional gastrointestinal flora. The mice were housed in static shoebox-type cages that had no filter tops or had molded polyester bonnets or Reemay filters that were 17.2 in^2 (Micro-Isolator™ [Lab Products, Seaford, Del.]) or 29.0 in^2 (Micro-Barrier [Allentown Caging Equipment Co., Allentown, Pa.]) filter lids. Cages contained either 850 cm^3 of hardwood chips or 850 cm^3 of pine shavings. Mean weekly ammonia levels in control (open) cages and cages with polyester bonnets were < 2.0 ppm. Mean weekly levels for Micro-Isolator™ and Micro-Barrier cages housing mice on hardwood bedding were 139.1 ppm and 162.8 ppm, respectively, with some cages reaching > 200 ppm by 6 days. Both mouse strains yielded similar responses, and results from pine shavings were similar to those from hardwood chips.

Huerkamp and Lehner (12) reported on cage micro-environments of Hsd:ICR female mice housed in static cages or three different types of ventilated caging. The cages contained 200 g of either corncob bedding alone or corncob bedding mixed with an ammonia-inhibiting substance. The mice were infected with *Proteus mirabilis*. Ammonia concentrations in cages housing 5 mice on 200 g of corncob bedding in static cages were > 100 ppm at the end of 7 days. Static cages containing a breeding pair of mice with offspring on corncob bedding yielded a mean ammonia concentration of 98 ppm. Perkins and Lipman (13) studied eight bedding types including those in our study, although the brands were different in some cases. They examined the micro-environments of DBA/1J mice housed in static isolator-type cages with polyester Reemay filters. Intestinal flora were undefined, so the mice may have been infected with *Proteus* spp. Each cage was supplied with 850 cm^3 of bedding, irrespective of type. At the end of 7 days, mean ammonia concentrations exceeded 50 ppm for all but corncob and virgin cellulose bedding. Mean ammonia levels for aspen and pine shavings exceeded 300 ppm at 7 days. Differences among bedding types in in-cage temperatures, RH, and CO_2 concentrations were not found.

A few reports have described the effects of exposure to gaseous ammonia on laboratory rodents, principally rats. Among the consequences reported have been rat mortality (14), depressed in vivo and in vitro immune responses in guinea pigs (15), and decreased concentration-dependent running (on a wheel) in Long-Evans rats and

Swiss mice (16), with rats being more affected than mice. Cessation of ciliary activity in rats has been reported after exposure to < 10 ppm of ammonia (17). Coon and colleagues exposed Sprague-Dawley and Long-Evans male and female rats to several concentrations of ammonia, either repeatedly or continuously (18). Repeated exposure (8 h daily, 5 days per week, for 6 weeks) to 155 or 770 ppm of ammonia resulted in no discernable toxic effects. A few rats had nonspecific inflammatory changes in the lungs. Continuous exposure to 40, 127, 262, 455, or 470 ppm lasted for 90 or 114 days. Of the rats exposed to 262 ppm, 25% had mild nasal discharge, no gross lesions at necropsy, and nonspecific changes in lungs and kidneys that were "difficult to relate specifically to ammonia inhalation." Continuous exposure to > 400 ppm of ammonia resulted in the death of 32 of 51 rats by 25 days of exposure and of 50 of 51 by day 65. The rats were mildly dyspneic and had nasal irritation. Continuous exposure to 470 ppm resulted in the death of 13 of 15 rats. Microscopic evaluation revealed focal and diffuse interstitial pneumonitis, calcification of renal tubular and bronchial epithelia, proliferation of renal tubular epithelium, myocardial fibrosis, and fatty changes in the liver. Similar changes were noted in dogs, rabbits, guinea pigs, and squirrel monkeys, but were also present in control animals, although the lesions were less severe. Rabbits in the high-dose continuous exposure study had opacity over one-fourth to one-half of their corneas. This finding was the basis for our microscopic examination of eyes in a recently concluded study of floor space needs of C57BL/6J mice. Mice exposed to 200 to > 400 ppm of ammonia had no visible eye lesions (manuscript submitted). Our removal of the NOD mice housed on CareFRESH Ultra in the first experiment reported here was arbitrary and based on anecdotal evidence that ammonia concentrations > 200 ppm are noxious for mice. The present bedding experiments were performed before the C57BL/6J housing density results were known. However, we do not know whether there is variation among mouse strains in their tolerance to ammonia.

Accidental exposure of humans to very high concentrations of ammonia can result in extensive thermal burns on the lips, conjunctival and corneal opacities, and edematous and congested lungs with areas of hemorrhage (19). However, exposure to concentrations as low as 20 ppm can cause discomfort and conjunctival hyperemia (20). Standards put forth by the National Institute of Occupational Safety and Health (NIOSH) indicate that workplace exposure to ammonia should not exceed 25 ppm over 8 h or 35 ppm over a 15-min period (21). However, there are two factors that can substantially reduce human exposure to ammonia in animal facilities. First, when filter tops are removed from rodent cages there is an immediate dilution effect by mixing with ambient air. Second, as is the case with Mus m 1 allergen exposure in mouse rooms (22), exposure can be greatly reduced by husbanding rodents on ventilated tables.

The early literature that addressed the noxious effects of low ammonia concentrations on rodents must be considered in the context of their microbial status at the time. Infectious diseases of the respiratory tract, specifically *Mycoplasma pulmonis* infection in rats, have been reported as cofactors in ammonia toxicity (23). Gamble and Clough (24) referred to some laboratory species as being unsuitable for studying inhalant toxicity because of their "natural incidence of abnormal respiratory histology" and suggested that this phenomenon might reflect the standard of husbandry in animal facilities. Fortunately, many of the previously common infectious agents of rodents are present at lower frequency now than two decades ago. Therefore, contemporary rodents may be able to tolerate higher in-cage ammonia concentrations in the absence of such exacerbating cofactors.

There are no accepted standards for rodent exposure to ammonia. We have examined nasal passages and eyeballs of mice exposed to higher ammonia concentrations than reported here and have not seen any lesions (Richard Smith, personal communication). In the absence of data indicating what ammonia concentrations might be noxious for mice, we may need to defer to the OSHA standards mentioned earlier. In light of cage micro-environment results, especially ammonia levels, we found that all bedding types that we evaluated, except for CareFRESH Ultra, were acceptable choices for use in static cages and that the practice of changing bedding every 2 weeks would likewise be acceptable. Ammonia concentrations in ventilated cages housing mice on CareFRESH Ultra bedding were at least an order of magnitude lower than those in static cages, so this bedding material may be successfully used in ventilated caging systems.

The ultimate choice of bedding material may depend on a variety of factors, including the purpose of the study in which the animals will be used. For instance, a multi-center consortium that will be measuring the effects of various drug interventions on a mouse model of aging recently requested our advice. The center directors realized that the three participating institutions were using different bedding materials and that pine shavings (used at one of the centers) might alter their results. As a consequence, the three centers compared the characteristics of readily available bedding materials and chose a single bedding for their experiments.

Acknowledgments

We thank Dr. Ralph Bunte for microscopic evaluation of nasal passages and Dr. Beverly Paigen, Dr. Muriel Davisson, and Dr. Kevin Flurkey for helpful comments during manuscript preparation. This research was funded in part by grant RR12552 from the National Center for Research Resources and by internal TJL funds.

References

1. **Kraft, L. M.** 1980. The manufacture, shipping and receiving and quality control of rodent bedding materials. Lab. Anim. Sci. **30:**366-376.
2. **Reeb, C. K., R. B. Jones, D. W. Bearg, H. Bedigian, D. D. Myers, and B. Paigen.** 1998. Microenvironment in ventilated animal cages with differing ventilation rates, mice populations, and frequency of bedding changes. Contemp. Top. Lab. Anim. Sci **37:**43-49.
3. **Port, C. D. and J. P. Kaltenbach**. 1969. The effect of corncob bedding on reproductivity and leucine incorporation in mice. Lab. Anim. Care **19:**46-49.
4. **Bazille, P. G., S. D. Walden, B. L. Koniar, and R. Gunther**. 2001. Commercial cotton nesting material as a predisposing factor for conjunctivitis in athymic nude mice. Lab Anim. (N.Y.) **30:**40-42.
5. **Sanford, A. N., S. E. Clark, G. Talham, G. Sidelsky, and S. E. Coffin**. 2002. Influence of bedding type on mucosal immune response. Comp. Med. **52:**429-432.
6. **Vessel, E. S.** 1967. Induction of drug-metabolizing enzymes in liver microsomes of mice and rats by softwood bedding. Science **157:**1057-1058.
7. **Vessel, E. S., C. M. Lang, W. J. White, G. T. Passananti, R. N. Hill, T. L. Clemens, D. K. Liu, and W. D. Johnson**. 1976. Environmental and genetic factors affecting the response of laboratory animals to drugs. Fed. Proc. **35:**1125-1132.
8. **Sabine, J. R.** 1975. Exposure to an environment containing the aromatic red cedar, *Juniperus virginiana*: procarcinogenic, enzyme-inducing and insecticidal effects. Toxicology **5:**221-235.
9. **Cunliffe-Beamer, T. L., L. C. Freeman, and D. D. Myers**. 1981. Barbiturate sleep time in mice exposed to autoclaved or unautoclaved wood beddings. Lab. Anim. Sci. **31:**672-675.
10. **Davey, A. K., J. P. Fawcett, S. E. Lee, K. K. Chan, and J. C. Schofield.** 2003. Decrease in hepatic drug-metabolizing enzyme activities after removal of rats from pine bedding. Comp. Med. **53:**299-302.
11. **Corning, B. F. and N. S. Lipman.** 1991. A comparison of rodent caging systems based on microenvironmental parameters. Lab. Anim. Sci. **41:**498-503.

12. **Huerkamp M. J. and N. M. Lehner**. 1994. Comparative effects of forced-air, individual cage ventilation or an absorbent bedding additive on mouse isolator cage microenvironment. Contemp. Top. Lab. Anim. Sci. **33:**58-61.
13. **Perkins, S. E. and N. S. Lipman**. 1995. Characterization and quantification of microenvironmental contaminants in isolator cages with a variety of contact beddings. Contemp. Top. Lab. Anim. Sci. **34:**93-98.
14. **Appelman, L. M., W. F. ten Berge, and P. G. Reuzel**. 1982. Acute inhalation toxicity study of ammonia in rats with variable exposure periods. Am. Ind. Hyg. Assoc. J. **43:**662-665.
15. **Targowski, S. P., W. Klucinski, S. Babiker, and B. J. Nonnecke**. 1984. Effect of ammonia on in vivo and in vitro immune responses. Infect. Immun. **43:**289-293.
16. **Tepper, J. S., B. Weiss, and R. W. Wood**. 1985. Alterations in behavior produced by inhaled ozone or ammonia. Fund. Appl. Toxicol. **5:**1110-1118.
17. **Dahlman, T.** 1956. Mucous flow and ciliary activity in the trachea of healthy rats and rats exposed to respiratory irritant gases. Acta. Physiol. Scand. **36(Suppl 23):**1-158.
18. **Coon, R. A., R. A. Jones, L. J. Jenkins, and J. Siegel**. 1970. Animal inhalation studies on ammonia, ethylene glycol, formaldehyde, dimethylamine, and ethanol. Toxicol. Appl. Pharmacol. **16:**646-655.
19. **Chao, T. C. and D. S. Lo.** 1996. Ammonia gassing deaths—a report on two cases. Singapore Med. J. **37:**147-149.
20. **Vigliani, E. C. and N. Zurlo**. 1956. Experiences of the Clinical Del Lavoro with maximum allowable concentrations of industrial poisons. AMA Arch. Ind. Health **13:**403.
21. http://www.cdc.gov/niosh/pel88/7664-41.html.
22. **Schweitzer, I. B., E. Smith, D. J. Harrison, D. D. Myers, P. A. Eggleston, J. D. Stockwell, B. Paigen, and A. L. Smith.** 2003. Reducing exposure to laboratory animal allergens. Comp. Med. **53:**346-351.
23. **Broderson, J. R., J. R. Lindsey, and J. E. Crawford.** 1976. The role of environmental ammonia in respiratory mycoplasmosis of rats. Am. J. Pathol. **85:**115-130.
24. **Gamble, M. R. and G. Cough.** 1976. Ammonia build-up in animal boxes and its effect on rat tracheal epithelium. Lab. Anim. **10:**93-104.