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Title

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Permalink

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Journal

Journal of the American Association for Laboratory Animal Science, 55(3)

ISSN

1559-6109

Authors

Carbone, Elizabeth T

Kass, Philip H

Evans, Kristin D

Publication Date

2016

Peer reviewed

Feasibility of Using Rice Hulls as Bedding for Laboratory Mice

Elizabeth T Carbone,^{*1} Philip H Kass,² and Kristin D Evans³

Factors that are considered when selecting laboratory mouse bedding include animal health and comfort, cost, effects on personnel, and bioactive properties. Corn cob is economical and facilitates low intracage ammonia but has undesirable influences on some endocrine studies. Rice hulls are an economical material that has not been well characterized as a bedding substrate. In this pilot study, we compared various aspects of bedding performance of rice hulls and other materials. On a per-volume basis, rice hulls were less absorbent than was corn cob bedding. Rice hulls had higher odds than did corn cob or reclaimed wood pulp of having moisture present at the bedding surface. The results of the absorbency tests coupled with the results of preliminary monitoring of intracage ammonia raised concern about the ability of rice hulls to control ammonia levels sufficiently in cages with high occupancy. However, ammonia was negligible when cages contained 5 young adult female mice. The relative expression of 3 cytochrome p450 genes was compared among mice housed on rice hulls, corn cob, reclaimed wood pulp, or pine shavings. The expression of Cyp1a2 was 1.7 times higher in the livers of mice housed on rice hulls than on pine shavings, but other differences were not statistically significant. This study provides information on the merits of rice hulls as laboratory mouse bedding. Their relatively poor moisture control is a major disadvantage that might preclude their widespread use.

Abbreviations: Cq, quantification cycle; RWP, reclaimed wood pulp.

Several types of mouse bedding are commercially available. Corn cob bedding is economical and facilitates low intracage ammonia.³⁴ However, neither mice nor rats prefer corn cob over aspen,²⁰ and corn cob influences outcomes in certain types of research.^{22,39} Corn cob contains tetrahydrofurandiols, linoleic acid derivatives with estrogenic properties, which disrupt mating behavior in rats²⁶ and confound some neuroendocrine studies.²¹ In addition, the mycotoxin zearalenone has been found in commercial corn cob and leads to delayed vaginal opening in mice.⁴⁴

Rice hulls are the outer husk of rice that is removed during processing and comprise 20% of the weight of the rice crop.¹⁴ Despite some industrial uses as biofuel and livestock litter, rice hulls are a relatively low-value side product of the rice industry.^{14,24,40} Although the hulls are used as bedding for pet rodents and in laboratory animal facilities in India (where they are called paddy husk), few published articles characterize this product. One bedding survey found rice hulls to be noncytotoxic; however, they moderately induced Cyp1a1, a cytochrome p450 enzyme, in a hepatoma cell line.³³ This result raises the possibility that rice hulls might confound research involving hepatic drug metabolism.

In the current study, rice hull bedding was evaluated with regard to absorbency, daily mouse health checks, and cytochrome induction. We hypothesized that rice hulls would be less absorbent relative to mass and volume than either corn cob or recycled wood pulp (RWP) bedding and that, after the application of liquid, the surface of rice hull bedding would dry more slowly than those of the other 2 beddings. Mice were observed for evidence of impaired health or reproduction as they grew and bred on the test beddings. Lastly, the expression of hepatic

cytochromes was compared across 4 bedding groups (rice hulls, corn cob, RWP, and pine shavings) by using real-time RT-qPCR analysis. We hypothesized that gene expression would be lower in the rice hulls group than in the pine shavings group.

Materials and Methods

Mice. The study population comprised 60 outbred CD1 mice (50 female mice [age, 3 wk]; 10 male mice [age, 8 wk]), which were received from the outbred breeding colony (UC Davis) in 2 cohorts. All procedures were part of an IACUC-approved protocol. Mice were housed in polycarbonate IVC (Optimice, Animal Care Systems, Centennial, CO). In this system, driven by the negative pressure of the room's central exhaust, air flows passively from the room through a front filter into the cage and then through a back filter into the central exhaust plenum. The central exhaust is programmed so that airflow through the exhaust hose is at least 40 ft³/min, which is equivalent to at least 20 air changes hourly at the cage level. The exhaust hose airflow was verified with an anemometer, but airflow at the cage level was not verified. All cages, except for the modified cages used for ammonia monitoring, were autoclaved prior to use. Mice were provided with unrestricted access to irradiated feed (2918, Harlan, Madison, WI) and chlorinated water in a pouch with a valve (Hydropac, Lab Products, Seaford, DE). Except during the ammonia-monitoring phase, each cage was enriched with 2 squares of cotton nesting material (Nestlets, Ancare, Bellmore, NY). Cage manipulations and changes were done in a cage-change station (Allergard, Nuair, Plymouth, MN).

The study took place at an AAALAC-approved facility. Mice were maintained in a room with a 14:10-h light:dark cycle, and relative humidity of 30% to 70%. Over the study period, the temperature ranged from 64 to 75 °F (17.9 to 23.9 °C). Each rack includes one cage with 2 sentinel mice, which are exposed to the soiled bedding of the colony mice. Quarterly testing of soiled-

Received: 07 Jul 2015. Revision requested: 31 Jul 2015. Accepted: 09 Sep 2015.
University of California, Davis, ¹Campus Veterinary Services; ²Population Health and
Reproduction; and ³Mouse Biology Program, Davis, California.
^{*}Corresponding author. Email: etcarbo@gmail.com.

bedding-exposed colony sentinels confirmed that the colony was free of the following pathogens during the study period: mouse hepatitis virus, Sendai virus, pneumonia virus of mice, mouse parvovirus, minute virus of mice, *Mycoplasma pulmonis*, *M. arthritidis*, Theiler murine encephalomyelitis virus, reovirus 3, lymphocytic choriomeningitis virus, ectromelia virus, epizootic diarrhea of infant mice, mouse adenovirus types 1 and 2, mouse norovirus, and *Helicobacter* spp.

Daily health observations. All mice were checked daily for signs of morbidity (quiet attitude, ruffled fur, hunched posture, squinted eyes, and external lesions). All cages were photographed daily during the first 14 d of the study and at the end of each cage-change interval. Subjective observations of cage wetness, viewed from above and below the cage, were noted in a daily log by a nonblinded observer. After 2 wk on test beddings, 24 female mice (age, 5 wk; $n = 6$ per group for corncob, rice hulls, RWP, and pine shavings) were euthanized for tissue collection, and their stomach contents were examined grossly.

Breeding. After the initial 2-wk period of acclimation and ammonia monitoring, 3 cages were prepared for each of 3 types of bedding—corncob, rice hulls, and RWP—and 1 male mouse was placed in each cage. Random 5-wk-old female mice were selected to form monogamous breeding pairs with male mice on the bedding type to which both mice were acclimated. Female mice were checked daily until copulatory plugs were noted. Sires were removed from cages on the day that the pups were born. On day 21 after parturition, all female pups and a maximum of 4 male pups per litter were weaned and moved to clean cages.

Test beddings. Four types of bedding were used: 1/4-in. corncob (Bed-o’Cobs, Anderson, Maumee, OH), rice hulls (packed by Frontier Ag, Davis, CA), RWP (CareFRESH, Absorption Corporation, Ferndale, WA), and pine shavings (Living World, Rolf C Hagen Corporation, Mansfield, MA). The 60 mice received from the breeding colony were housed on 1/4-in. corncob at their facility of origin. Corncob was purchased from a laboratory vendor, rice hulls came from a local feed and pet supply store (River Valley Feed and Pet Store, Rio Linda, CA), and RWP and pine shavings were bought from a major pet-supply store. RWP was removed from its package and shaken loose before autoclaving. All bedding was autoclaved prior to use in cages and for absorbency or moisture testing. When measured, RWP was gently packed in an attempt to standardize density. Bedding was added to individual cages to the following volume and depth: corncob and rice hulls, 500 cm³ (depth, approximately 1/4 in.); RWP and pine shavings, 1000 cm³ (depth, approximately 1 in.). Cages were changed every 14 d.

Bedding absorbency. In a method modified from a previous study,⁴ 500 cm³ of corncob, RWP, or rice hulls was measured in a beaker. This process was repeated 10 times per bedding, and group means were determined. A mean-weight volume of 500 cm³ of each bedding was divided into 10 equal-weight portions of 50 cm³ in volume. The RWP was packed down gently for the measurement. Each 50-cm³ sample was placed in a plastic container (Rubbermaid, Wooster, OH) and flooded with 100 mL of 1% saline (made by dissolving 10 g table salt in 1 L municipal tap water) so that the bedding was fully immersed; 10 samples of each bedding were left to soak for 1 h. After soaking, each container was emptied into a sieve (Good Cook, Bradshaw International, Rancho Cucamonga, CA). The sieve was agitated 20 times to shake off excess water, and the wet bedding was weighed in the sieve. The volume of 1% saline absorbed was calculated by subtracting the dry mass of the bedding sample from the wet mass. Absorbency by volume and absorbency by

mass were calculated from the sample volume, sample mass, and volume of saline absorbed.

Surface moisture. Empty cages were filled with 500 mL corncob bedding, 500 mL rice hulls, or 1 L RWP ($n = 33$ cages per group, 3 per bedding type per time point). The RWP was gently packed down in the measuring cup and again once in the cage. Municipal water dyed with food coloring was drawn into a 1-mL syringe and used to make 6 wet areas in each cage, by holding the syringe just above the bedding surface and discharging 0.5 mL of liquid per area. The locations of the aliquots were standardized among cages (Figure 1 A through D). Surface moisture at baseline and hourly thereafter for 10 h was assessed in 3 cages per bedding type. Except for the cages assessed at baseline, all cages were covered with a lid and placed on the ventilated cage rack until their designated time point. Cages did not contain feed, water, or nesting material. Surface moisture was determined by using cobalt chloride test paper (Indigo Instruments, Niagara Falls, NY), which changes color on contact with water. A 2.5-cm strip of test paper was placed on the surface of a wetted area, and a 45-g weight on top of the test paper to hold it in contact with the bedding particles. The contact surface of the weight was a flat plastic surface of 2 cm in diameter. After 5 s, the weight and test paper were removed, and the test paper was photographed (iPhone 5s, Apple, Cupertino, CA). The photographs were presented in random order to a blinded observer, who rated them as positive (color change) or negative (no color change; Figure 1 E). The proportion of positive strips was calculated for each cage.

Ammonia monitoring. At the start of the study, 3-wk-old female mice were randomly assigned 5 per cage to cages containing corncob ($n = 3$ cages), rice hull ($n = 3$), RWP ($n = 2$), or pine shaving ($n = 2$) bedding. Each of these 10 cages was modified with a port on 1 side to allow placement of a tube for air sampling. When not in use, the port was covered with plastic paraffin film. Intracage ammonia levels were measured in the occupied cage by using a multigas analyzer (MultiRAE IR, RAE Systems, San Jose, CA). To take a reading, the sampling tube was placed through the port until the tip rested 4 cm from the cage wall and 6.5 cm from the cage bottom. The monitor took continuous readings for 3 min, based on the manufacturer’s recommendations for ammonia detection, and the peak ammonia value was recorded. Air samples were taken from a single clean cage of each bedding type before mice were added, and all cages were sampled on days 7, 10, and 14. During this 14-d period, the 10 male mice were acclimating in cages with corncob, rice hull, or RWP bedding. The cutoff ammonia level that would require a cage change was set at 25 ppm.

Cytochrome quantification. Female mice (age, 5 wk) were randomly selected for gross necropsy and tissue collection after being housed on a test bedding (rice hulls, corncob, RWP, or pine shavings, $n = 6$ per bedding) for 14 d. The mice were euthanized by carbon dioxide inhalation. Liver tissue was collected immediately, placed in RNA stabilization agent (RNAlater, Qiagen, Valencia, CA), and then stored at -80°C .

The 24 liver samples were thawed and RNA was extracted (RNeasy Mini Kit, Qiagen). Approximately 20 mg liver tissue was placed in 600 μL lysis buffer and homogenized (Rotor-stator homogenizer, Omni International, Tulsa, OK). The extraction was completed according to the protocol supplied with the kit. RNA concentration was measured by using a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE). Reverse transcription was done by using a commercial kit (High-Capacity cDNA Reverse Transcription Kit, Life Technologies, Grand Island, NY) according to the manufacturer’s protocol.

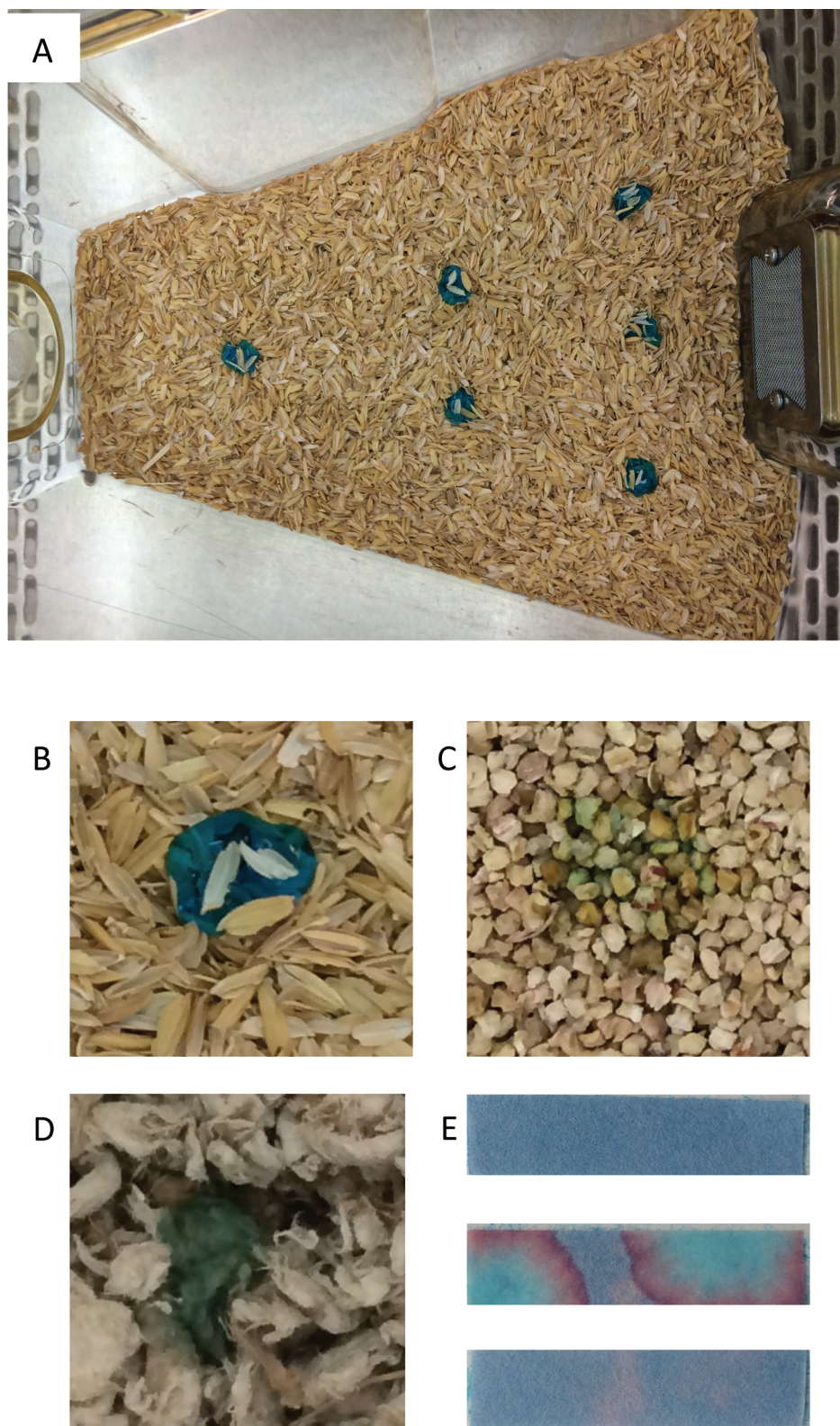


Figure 1. Placement of volumes of blue-colored water on the bedding surface and appearance of moisture-sensitive cobalt chloride strips. (A) Standardized locations of 6 volumes of water, shown here on rice hulls. Immediately after placement, water (B) formed a bleb on rice hulls but was absorbed by (C) corncob bedding and (D) reclaimed wood pulp. After contact with wetted spots, (E) cobalt chloride strips were graded as negative (top) or positive (strong, middle; weak, bottom) for color change.

The quantity of RNA used for the reverse transcription was standardized to 1 μ g. For reverse transcription, the thermal cycler (Tetrad 2, BioRad, Hercules, CA) was set to 25 $^{\circ}$ C for 10 min, 37 $^{\circ}$ C for 120 min, and 85 $^{\circ}$ C for 5 min.

Commercially available primer-probe assays for target and reference genes were purchased (TaqMan Real Time PCR Assays, Life Technologies). FAM-labeled target genes (catalog no. 4331182, Life Technologies) included *Cyp1a1* (Mm00487218_

m1), Cyp1a2 (Mm00487224_m1), Cyp2c37 (Mm00833845_m1), and Cyp3a11 (Mm00731567_m1). The VIC-labeled reference gene (catalog no. 4448489) was Pgk1 (phosphoglycerate kinase 1, Mm00435617_m1).

For real-time RT-qPCR, wells in a 384-well plate were each filled to a total volume of 10 μ L with master mix (Quantitect Multiplex PCR MasterMix, Qiagen), reference assay, target assay, nuclease-free water, and cDNA. The amplification was performed on a thermal cycler (7900HT Fast Real-Time PCR System, Life Technologies) for 40 cycles of 15.5 min at 95 °C and 1 min at 60 °C. Standard curves were constructed for each target gene by running dilutions of the gene (in duplicate wells) in tandem with a fixed concentration of Pgk1. Four dilutions, spanning a total dynamic range of 27-fold, were made for each target gene assay, by using the cDNA template from a single mouse sample. No-template controls and genomic DNA template controls were run on the same plate as the dilutions. For the analysis of bedding effect, all samples were run in triplicate wells with a target gene paired with the reference gene.

The quantification cycle (Cq) was determined by using specialized software (SDS 2.4 and SDS RQ Manager, Life Technologies). For the standard curves, Cq was on the y -axis and the log of the relative template cDNA concentration was on the x -axis. For each curve, amplification efficiency (E) was calculated for the target and reference genes by using the formula $E = 10^{(-1/\text{slope})}$.³⁵

Data analysis. Analyses were performed by using R (version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria) or Stata (version 13.1, StataCorp, College Station, TX). Absorbency data distributions were compared by using the Kruskal–Wallis test and pairwise Mann–Whitney U tests. For surface moisture, multilevel logistic regression was used to estimate the main effects of bedding type while controlling for time (as a categorical variable) and location within cage; cage was treated as a random effect. Results are presented as odds ratios and 95% confidence intervals. For the analysis of bedding effects on cytochrome expression, Δ Cq was calculated, and values were excluded as outliers when their standardized residuals had an absolute value greater than 2.5. Normality of data was verified by using the Shapiro–Wilk test. Δ Cq values were compared by using mixed-effects ANOVA and pairwise comparisons; in this model, bedding group and technical replicate were fixed effects and mouse identity was a random effect. For significant pairwise comparisons, the expression ratio was calculated as $2^{-\Delta\Delta\text{Cq}}$.²⁵ For all analyses, P values less than 0.05 were considered significant. Posthoc P values underwent Bonferroni–Holm adjustment.

Results

Daily health observations. In cages with male mice during the first 14 d of study, areas of wet bedding at the cage bottom were perceived to be more frequent and larger in the cages with rice hulls than in cages with corncob or RWP bedding (Figure 2).

All 9 breeding pairs each produced a litter, ranging from 9 to 18 pups. Including pups, a total of 167 mice were involved in the study; 162 of these mice had no signs of morbidity (quiet attitude, ruffled fur, hunched posture, squinted eyes, or external lesions) on daily observation. One pup from a litter of 18 pups housed on rice hull bedding was found dead at 2 d of age. In addition, 4 littermates housed on RWP were noted to have progressively scruffy hair coats and squinted eyes for 3 consecutive days and were euthanized. An etiology for these nonspecific signs was not found on gross necropsy or histopathology.

Mice were observed manipulating rice hulls with their mouths after cage changes. However, bedding material was not grossly noted in the gastric contents of any mouse. Microscopic analysis was not performed.

Bedding absorbency. The weight (mean \pm 1 SD) of 500 cm^3 of bedding was 210.0 \pm 4.1 g for corncob, 59.2 \pm 2.1 g for rice hulls, and 36.1 \pm 1.4 g for RWP (Figure 3). According to the group mean bedding weights, the saline saturation test of absorbency was performed on 10 corncob samples weighing 21.0 g each, 10 rice hull samples weighing 5.9 g each, and 10 RWP samples weighing 3.6 g each; for all 30 samples, the volume was assumed to be 50 cm^3 . Differences in group means for density, absorbency by volume, and absorbency by mass were significant ($P < 0.001$). Pairwise comparisons for bedding density were all significant ($P < 0.001$). Absorbency by volume (mean \pm 1 SD) was 0.51 \pm 0.02 mL/cm^3 for corncob bedding, 0.42 \pm 0.03 mL/cm^3 for rice hulls, and 0.41 \pm 0.02 mL/cm^3 for RWP ($n = 10$ for each bedding). Pairwise P values for absorbency by volume were: corncob bedding compared with rice hulls, $P < 0.001$; corncob bedding compared with RWP, $P < 0.001$; and rice hulls compared with RWP, $P = 0.273$. Absorbency by mass (mean \pm 1 SD) was 1.20 \pm 0.05 mL/g for corncob bedding, 3.52 \pm 0.25 mL/g for rice hulls, and 5.68 \pm 0.24 mL/g for RWP ($n = 10$ for each bedding). For all pairwise comparisons for absorbency by mass, P values were less than 0.001.

Surface moisture. The odds of positive moisture-detecting strips (Figure 4) were significantly higher with rice hulls than with corncob (odds ratio, 7402; 95% confidence interval, 1143 to 47,847; $P < 0.001$) or RWP (odds ratio, 35; 95% confidence interval, 12 to 108; $P < 0.001$). The odds of positive strips were significantly higher with RWP than with corncob (odds ratio, 19; 95% confidence interval, 45 to 1060; $P < 0.001$).

Ammonia monitoring. Of the 10 cages monitored, 7 were opened once during the 14-d period to replace feed or water. On days 0, 7, and 10, the ammonia level in all cages was 0 ppm. On day 14, ammonia was 0 or 1 ppm in all cages.

Cytochrome quantification. RNA concentrations of samples ranged from 0.03 to 2.89 $\mu\text{g}/\mu\text{L}$, with a median of 0.53 $\mu\text{g}/\mu\text{L}$. For the least concentrated sample, only 0.3 μg of RNA was reverse-transcribed (rather than 1.0 μg) due to volume constraints. A standard curve could not be made for the Cyp1a1 gene because Cq values were excessively high or could not be determined by the software, although the amplification efficiency of the reference gene was 1.97. Amplification efficiencies for the other standard curves were as follows: Cyp1a2, 2.03 (target) and 1.99 (reference); Cyp2c37, 1.91 (target) and 1.89 (reference); and Cyp3a11, 1.96 (target) and 2.16 (reference). Ideal efficiency is 2.00.

For each target gene, 72 Δ Cq values were calculated (24 mice, 3 replicates each); 5 values were excluded as outliers. The mixed-effects ANOVA yielded a significant F value for Cyp1a2 ($n = 70$) and nonsignificant F values for Cyp2c37 ($n = 71$) and Cyp3a11 ($n = 70$). The analysis controlled for the effect of technical replicates. For pairwise comparisons, the comparison of Cyp1a2 expression between mice housed on rice hulls (Δ Cq, -1.362 ± 0.348) and those on pine shavings (Δ Cq, -0.647 ± 0.496) yielded the only significant difference ($P = 0.012$). The combined standard deviation for $\Delta\Delta$ Cq was 0.606. The mean Cyp1a2 expression ratio of the rice hull group relative to the pine shavings group (calculated as $2^{-\Delta\Delta\text{Cq}}$) was 1.692 (range, 1.112 to 2.576).

Discussion

This project was designed as a pilot study with broad objectives. Experiments within the study focused on the aspects



Figure 2. Appearance of cages containing corncob bedding (CC), rice hulls (RH), or reclaimed wood pulp (RWP) at the end of a cage-change interval. Cages were occupied by 2 or 3 male mice. Moisture collection was subjectively greatest beneath the rice hull bedding.

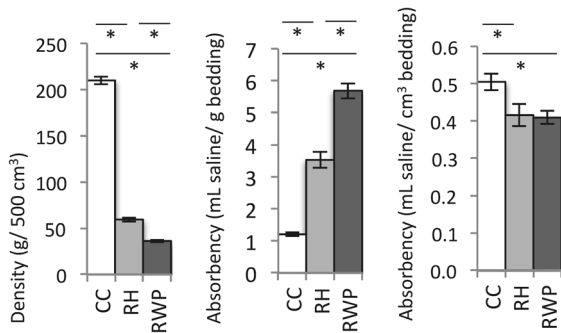


Figure 3. Physical characteristics of corncob bedding (CC), rice hulls (RH), and reclaimed wood pulp (RWP) ($n = 10$ for all groups). Data are presented as mean values; error bars, 1 SD. *, Significant ($P < 0.001$) difference between values.

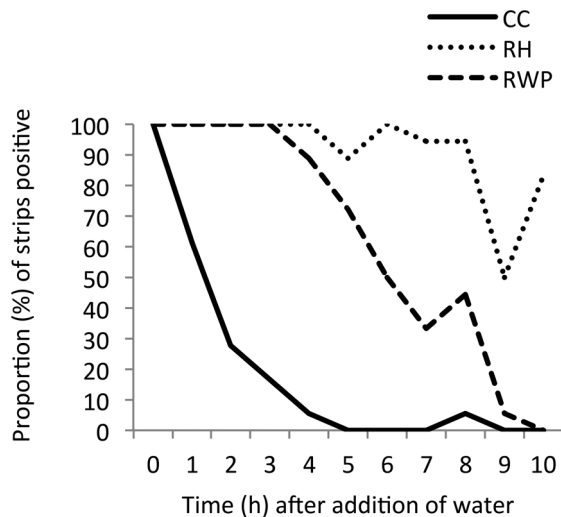


Figure 4. The persistence of surface moisture on corncob (CC), rice hull (RH), and reclaimed wood pulp (RWP) bedding for 10 h after application of aliquots of water at 0 h ($n = 3$ cages per bedding per time point). Cobalt chloride strips were considered positive when they showed color change, indicating contact with moisture at the bedding surface.

of bedding evaluation related to mouse health and effects on research. Rice hulls were less absorbent than was corncob or RWP on both a per-mass and per-volume basis. Using a novel method of assessing the persistence of moisture at the bedding surface, we found that the surface of rice hulls had much higher odds of being wet than did the surfaces of corncob or RWP after the administration of fixed volumes of water. Cytochrome p450 gene expression in the liver was generally comparable among

mice that had been housed on rice hulls, corncob, RWP, or pine shavings, although the gene expression of one cytochrome subtype was significantly higher in the rice hulls group than in the pine shavings group.

Mice were observed daily for signs of morbidity. Bedding was never noted to be adhered to the skin or eyes of mice of any age, and no mouse had gross external lesions during daily observations or at necropsy. The single spontaneous death of a neonate from a litter of 18 in a rice hull cage was not considered abnormal. Of the 4 euthanized littermates from a RWP cage, all underwent gross necropsies, and the tissues from 1 were examined histologically. No gross or histologic lesion was found to explain the nonspecific signs of illness (scruffy coat, squinting), but a bedding-related cause, such as cold stress secondary to wet bedding, cannot be ruled out. Further research with a larger sample size and complete gross necropsy and histopathology is needed to demonstrate that the morbidity and mortality rates are statistically equivalent across beddings. Although mice manipulated the bedding with their mouths when placed in clean cages, none of the 24 mice necropsied at the end of the first 14 d had any bedding in the stomach. No evidence from this study suggests that gastrointestinal impaction of rice hulls is a risk in mice with unrestricted access to food.

During the first 2 wk of the study, the wetness at the bottom of the cages holding male mice was subjectively greater in cages containing rice hulls than those with corncob or RWP (Figure 2). In this study, the moisture analysis was performed at the bedding surface of unoccupied cages rather than at the cage bottom. The subjective results imply that quantifying the moisture that percolates through the bedding to the cage bottom might yield significant results as well.

The unprocessed rice hulls used in this study cost less than did either the corncob and RWP beddings. On the basis of our facility's supply costs for commercially available bedding and the quantity of bedding used per cage, the per-cage bedding cost was \$0.02 for rice hulls, \$0.12 for corncob, and \$0.21 for RWP (data not shown). However, if commercial rice hull bedding labeled for laboratory rodents were to become available and thus subject to the processing and quality control standards of comparable bedding, the cost advantage of rice hulls likely would decrease.

The potential of the tested beddings to control moisture was characterized by determining absorbency and relative drying time. The benefits of moisture control for bedding are the maintenance of a dry substrate in contact with the animals and the reduction of fecal urease-producing bacteria that convert urea in waste to ammonia.^{17,41} Absorbencies were analyzed relative to mass and to volume. Across beddings of variable density, it is easier to standardize mass than volume, making absorbency

by mass an appealing parameter. However, in a previous study,⁴ 3 technicians filled cages with 2 different beddings to uniform depth rather than to uniform mass. If this reported procedure is consistent with common practice, absorbency by volume is a more meaningful parameter to estimate the absorptive capacity of the total bedding in cages. However, it is difficult to extrapolate from the reported study⁴ whether using depth to fill cages with bedding is universal. In addition, absorbency by volume is difficult to standardize for loose beddings, because the density and absorptive capacity of samples are highly dependent on the packing of the samples. To illustrate this point, the RWP packaging lists the volume of its contents in both compressed and expanded forms, and the expanded volume is more than twice that of the compressed volume.

In the current study, a single person gently packed the loose autoclaved RWP bedding to maximize standardization of density of this bedding across all experiments. Corncob and rice hull beddings were not compressible, so their densities were standardized easily. Bedding densities, absorbencies by mass, and absorbencies by volume are shown in Figure 3. The rank orders are inverse for density and absorbency by mass, with corncob the most dense and least absorbent by mass. In contrast, in the case of absorbency by volume, corncob was significantly more absorbent than were rice hulls and RWP, with rice hulls and RWP almost equivalent.

The properties of the saturated samples varied by bedding. Corncob granules retained their size and firmness, and relatively little saline was shaken off when the samples were agitated. Water droplets adhered to the exterior of the rice hulls, which retained their size and shape. RWP particles became soft and swollen and discharged a relatively high, variable amount of excess saline when agitated in a sieve. Given the variation in how the materials interacted with saline, it is unclear whether absorbency (as determined by a saturation test) meaningfully indicates how a bedding will control moisture in an occupied cage with regard to intracage ammonia and substrate dryness at the bedding surface.

The rate at which urine and water dry at the bedding surface may not necessarily be indicated by absorbency trials using saline saturation, although absorbency has often been determined in bedding studies.^{4,10,19,37} Mice are in physical contact with bedding at the surface, where retained moisture can contribute to dermatitis and impaired thermoregulation. Substrate that is wet or has fecal contamination is a risk factor for pododermatitis in rabbits, rodents, and avians.^{3,28} Because we were unable to locate a previously published method for measuring surface moisture, we designed a procedure to determine relative drying times of the beddings in a simulated applied situation.

The experiment was meant to assess relative rather than absolute drying time, because of the difficulty of replicating actual cage conditions. The urine load excreted onto the bedding in a cage depends on the biomass of cage occupants as well as their sex, strain, social status, and preferred location of excretion.^{11,47} Experimental variables affecting the results include the volume of water, the pressure with which the water is applied to the surface, the pressure at and duration for which the contact paper is held in contact with the bedding surface, and the criteria for classifying the contact paper as positive or negative. Altering any of these variables would diminish the reproducibility of absolute drying times but theoretically should not alter the relative relationships of drying times. To simulate the conditions of occupied cages, the same amount of bedding was used in this experiment as in the rest of the study, and the cages remained on the ventilated cage rack until the designated time point.

For the 10-h period after applying volumes of water to beddings, the odds of being wet were highest for rice hulls, intermediate for RWP, and lowest for corncob. Figure 4 shows the proportion of test strips read as positive (wet) at each time point. The poor drying ability of rice hulls was consistent with the initial behavior of the water when it was applied to the bedding surfaces—that is, the water added formed a bleb only on rice hulls (Figure 1); over subsequent time points, the bleb reduced in size or disappeared, and the liquid percolated through the rice hulls. The liquid then collected at the cage bottom, where it was visible from below the cage more consistently and persistently than for corncob or RWP bedding (Figure 2).

It can be surmised from this experiment that rice hulls are relatively hydrophobic. Indeed, the composition analysis of rice hulls indicates that they are high in lignin,^{14,40} a hydrophobic molecule. In contrast, RWP has higher contents of the hydrophilic molecules cellulose and hemicellulose, and most of the lignin is removed in the pulping process. Water clings to the hydrophilic molecules and is drawn into the material by capillary action. Corncob bedding, made from the woody ring component of the corncob, is advertised as having “sponge-like characteristics” and facilitating “bottom-up” absorption, but we were unable to find a more thorough description of the mechanism of absorbency.

The purpose of daily monitoring of intracage ammonia during the first 14 d of the study was to ensure that the study could proceed, with 14-d cage-change intervals, without the ammonia level surpassing a predetermined threshold. With an occupancy of 5 young adult female mice, ammonia remained at 0 to 1 ppm throughout the 14 d for all 4 beddings. Later in the study, when some cages contained multiple male mice or dams with litters, some cages developed an ammonia odor. Ammonia monitoring was reinitiated to ensure that the cages were changed should they reach the predetermined threshold of 25 ppm. Ammonia surpassed 25 ppm in some cages with rice hulls (data not shown). Cages with multiple male mice and dams with nursing litters have previously been associated with high intracage ammonia.⁴⁷ Because this later monitoring was not designed or executed as an experiment, these data were not analyzed further.

Although the safety limit of ammonia levels for mice is unknown, the National Institute for Occupational Safety and Health workday time-weighted average exposure limit for humans, 25 ppm, is often used as a guideline.^{5,10,38,42} Histologic lesions of the nasal passages depend on the concentration and duration of ammonia exposure. In a study that used a 28-d cage-change interval, mice exposed to a daily mean ammonia level of 32 ppm had no nasal histologic lesions, those exposed to a daily mean of 52 ppm showed degeneration of respiratory epithelium, and those exposed to a daily mean of 181 ppm had rhinitis.⁴⁷ In preference testing, mice showed no aversion to chambers with mean ammonia concentration of 110 ppm.¹⁶ It is important to note that ammonia levels can vary within a cage, being highest at the latrine sites; therefore measurements taken at a standardized single location may not reflect the true ammonia exposure experienced by mice as they move around the cage.^{12,13}

In the current study, rice hulls, RWP, and corncob showed differences in their ability to control of surface moisture but were equally effective at controlling ammonia with a cage population of young adult females. However, we suspect that with a higher urine load in the cage, the difference in surface moisture control might result in appreciable differences in intracage ammonia levels. This notion is consistent with the conclusions of

previous studies, which associated cage moisture with bacterial proliferation and increased intracage ammonia.^{4,15,29,37} In those studies, cage moisture was measured as relative humidity, which was increased with poorly absorbent bedding or leaked water bottles.^{4,15,29,37} When microbiologic load was measured directly in a study that compared different types of corncob bedding, bacterial load rather than absorbency by mass predicted the type of bedding that had the best ammonia control.¹⁰ Different RWP formulations have previously been shown to have poorer ammonia control than does corncob bedding.^{13,34,42} One study measured the ammonia level by determining the amount of free amino nitrogen in soiled bedding and found that sawdust had a higher ammonia concentration than did rice hulls, shredded paper, or corncob bedding, and the latter 3 products did not differ significantly in this regard.¹ Whether free amino nitrogen content and airborne ammonia can be compared directly is unknown. The airborne ammonia levels in cages containing rice hulls have not previously been tested, to our knowledge.

Increasing bedding volume has been correlated with decreasing intracage ammonia,³⁸ although the maximal volume in some IVC, such as those used in this study, is limited by the need to avoid blocking airflow filters located low in the cage. Studies comparing beddings of variable density and compressibility have based bedding volume on a standard volume^{13,34} or on facility standard operating procedures and bedding manufacturers' recommendations.⁴² In the current study, the volume of corncob bedding used was based on facility standard operating procedures and that for RWP on manufacturer recommendations. Because rice hulls, like corncob bedding, cannot be compressed or expanded, the volume used was matched to that of corncob bedding. Similarly, because pine shavings, like RWP, are low-density and compressible, the volume used was matched to that of RWP. Intracage ventilation might have been impaired due to the presence of compressible beddings and ground feed pellets piled in front of airflow filters.

Hepatic cytochrome p450 enzymes aid in the metabolism of both xenobiotics and endogenous compounds.⁵⁰ Substances that induce the activity of these enzymes generally do so by increasing gene transcription.⁷ The ability of test substances to induce cytochromes is often studied in the context of preclinical toxicity testing.⁴⁸ In laboratory animal science, compounds in the animals' environment that are capable of inducing enzymes are of interest because of their influence on research results. Softwood beddings with cytochrome-inducing compounds confound pharmacology research and reduce the efficacy of barbiturate anesthetics.^{8,46}

Previous studies have quantified bedding-associated cytochrome induction by measuring the end products of cytochrome-mediated enzymatic reactions and the protein content of hepatic microsomes. Some studies exposed mice or rats to test beddings simply by housing the animals on the bedding.^{8,9,46,49} One study compared this method of exposure with oral administration of extracted volatile compounds and found that oral administration caused more pronounced induction.²³ For example, the microsomal Cyp3a content increased 7.1-fold over control levels after oral administration but only 2.3-fold after contact exposure.²³ Another study concluded that *in vitro* enzymatic assays using bedding extracts and Hepa1 cells are more sensitive—and therefore better for screening—than are *ex vivo* enzymatic assays of liver tissue from animals housed on test bedding.⁴⁵ Hepa1 is a mouse hepatoma cell line with highly inducible Cyp1a1.³⁶

Using the Hepa1 assay, another study of 28 beddings ranked the relative inducing ability by bedding category, with soft-

woods causing the greatest induction of cytochrome enzymes, followed by hardwoods and grass-derived beddings including rice hulls (moderately inducing) and corncob (noninducing).³³ In light of the ability of various softwoods to reduce pentobarbital-induced sleep time, another study concluded that red cedar is the softwood that leads to the highest induction of cytochrome gene transcription, followed by white pine and then white spruce.⁸ In that study, autoclaving the softwood beddings did not reduce the effect of softwood bedding on sleep time.⁸ In contrast, a chemical analysis of bedding extracts found that autoclaving decreased the volatile organic compounds in softwood bedding.³¹ Another treatment—soft-hydrothermal processing of cedar by using steam and pressure to remove various volatile and aromatic compounds—successfully decreased the bedding's cytochrome-inducing activity.²³ Corncob was used as the negative control bedding for relative quantification in the present study because no cytochrome-inducing effect has been attributed to it.^{33,49}

Real-time RT-qPCR analysis can be used to quantify the relative transcription of cytochrome p450 genes, although (to our knowledge) it has not previously been used to evaluate the inducing effects of beddings. Prior studies have measured the effect of known pharmaceutical inducers of cytochrome p450 enzymes.^{2,27,32,43,48} Reported fold increases in one mouse study ranged from 2 fold (Cyp3a11, induced by dexamethasone) to 880 fold (Cyp1a1, induced by β -naphthoflavone).²⁷ qPCR analysis compares favorably with enzymatic assays and protein quantification assays for the assessment of cytochrome induction.^{2,48} Advantages of RT-qPCR analysis include its reproducibility and sensitivity over a wide range of cDNA concentrations.² RT-qPCR assays are fast, require less tissue than do other methods, and can be targeted (through primer specificity) to any cytochrome subtype of interest.⁴⁸

The choice of cytochrome subtypes as target genes for the present study was based on previous studies of bedding-associated induction of cytochrome enzymes. The only study to include rice hulls among the test beddings found that they moderately induced Cyp1a1 in the Hepa1 cell line;³³ we therefore included Cyp1a1 to see whether this finding was replicated by using real-time RT-qPCR analysis of *ex vivo* samples. Although Cyp1a1 is constitutively expressed at low levels in mouse and rat liver,^{2,6} this level of gene expression has been detected by using real-time RT-qPCR.² In the present study, we were unable to consistently detect the Cyp1a1 P450 subtype in any bedding group. We chose cytochromes Cyp1a2 and Cyp3a11 for analysis because their activity and content were increased by softwood beddings in previous studies.^{9,23} In addition, because one of the studies included a measure of Cyp2c content (the antibodies used could not distinguish between mouse cytochromes at the subtype level),²³ we included a Cyp2c subtype in the present study as well. We chose Cyp2c37 because it is the mouse homolog of human CYP2C9,³⁰ an important cytochrome for toxicity testing.⁷ Cyp2e1 was included in previous studies but not in the current study because this enzyme is known to be regulated by posttranscriptional and transcriptional mechanisms.² Cyp1a2, Cyp2c37, and Cyp3a11 are all expressed in the liver.⁶ Important mouse subtypes not evaluated here but which may be assessed in future studies include Cyp2e1, Cyp2b10, and Cyp2d9.³²

The reference gene used in the present study, Pgk1, was used successfully as a reference for real-time RT-qPCR analysis of Cyp1a2, Cyp3a11, and others and was stably expressed across animal subjects.⁴³ Other real-time RT-qPCR studies of mouse and rat cytochromes have used GAPDH or 18s rRNA as a reference gene.^{2,27,32,48} In future studies, the use of multiple reference

genes should be considered to increase the accuracy of target gene normalization.¹⁸

The relevance of the single significant result of the mixed-effects ANOVA—in the absence of a pattern across target genes or bedding groups—is difficult to interpret. For *Cyp1a2*, gene expression was 1.7-fold higher in the rice hull group than in the pine shavings group. This result might be consistent with the previous study in which rice hulls induced *Cyp1a1* transcription in Hepa1 cells,³³ given that both enzymes are members of the *Cyp1a* subfamily. In view of the known cytochrome-inducing ability of softwoods, we expected that exposure to pine shavings would increase the expression of all cytochrome enzymes. However, the shavings used in the current study may lack the induction activity that is typical of pine. According to the product packaging, the pine shavings used at our facility undergo “kiln-drying,” which “reduces the concentration of naturally occurring aromatic oils present in pine.” In addition, the pine shavings were autoclaved before use and, as mentioned earlier, a chemical analysis study found that autoclaving decreased the concentration of volatile organic compounds in softwoods.³¹ These multiple heat exposures might have reduced the bioactivity of the pine shavings used in the current study. In future studies, the use of validated positive and negative controls would facilitate interpretation of results.

Other possible reasons for the low number of significant results in this study include a true absence of a bedding-associated effect, a lack of sensitivity due to biologic variability, inappropriate target and reference gene selection, and issues with sample preparation. We believe that 14 d of exposure is sufficient for the bedding to exert any cytochrome-inducing effect, because previous studies found that a change in bedding can alter cytochrome activity within a few days.^{9,23,46} The use of an outbred mouse stock was—by definition—a source of biologic variability. However, we chose the CD1 stock for consistency with previous studies of real-time RT-qPCR evaluation of cytochrome induction.^{23,27,48}

We suspect that the relatively poor moisture control shown by rice hulls make them an impractical alternative to current commonly used beddings in most settings. If further research is pursued, the cage-change interval that maintains an acceptable level of ammonia for a particular population of mice should be determined. The interplay of microenvironmental variables would be analyzed more readily by using a caging system that monitors airflow at the cage level, in contrast to the passive airflow ventilated caging used in the current study. Monitoring of intracage humidity and microbiologic load of bedding would allow better characterization of ammonia formation on rice hulls. To investigate whether rice hulls (like corncob bedding) have hormonal bioactivity, analyzing them for tetrahydrofurandiols and zearalenone would be useful. Other topics for further research include the suitability of rice hulls in various caging types and with different rodent species, animal preference testing, the identification of potential biologic or chemical contaminants or intrinsic bioactive compounds of rice hulls, and the usefulness of rice hull bedding for particular animal models.

Acknowledgments

This study was supported in part by the Hands-on Education and Learning Program (HELP) of UC Davis' Mouse Biology Program. We thank the staff of the Mouse Biology Program, especially Brandon Willis and Jeff Evans (Murine Genetic Engineering Laboratory) and Vincent de Vera (Vivaria and Veterinary Care Group).

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