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Effects of Extruded Compared with Pelleted Diets on Laboratory Mice Housed in Individually Ventilated Cages and the Cage Environment

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The physical form of the diet fed to laboratory animals should be evaluated to reduce experimental variations and confounding factors. This 14-d study evaluated the effects of diet form (pelleted or extruded) on intracage ammonia concentrations, feed disappearance, body weight, cage weight, and the degree of cage soilage and whether these effects were influenced by strain or stock or sex. Mice (C57BL/6, ICR, and nude; age, 4 wk) were randomly assigned to 4 treatment groups representing pelleted and extruded diets from each of 2 vendors (pelleted diet groups, P1 and P2; extruded diet groups, E1 and E2). Intracage ammonia concentrations depended on strain or stock, diet, and day and were higher in cages housing nude mice that consumed P1. Diet type did not affect the weight of mice at the end of the study. Feed disappearance was dependent on diet type and mouse strain or stock and was greatest in the cages of mice that consumed P1. In addition, the greatest feed disappearance was seen with ICR mice, whereas the least was seen with C57BL/6 mice. Cages housing male nude mice had greater cage soilage than those housing female nude mice. The degree of cage soilage was influenced by diet type and day also. These results show that diet form and mouse strain or stock significantly affect intracage ammonia concentrations, feed disappearance, cage weight, and the degree of cage soilage.

Abbreviations: E, extruded; P, pelleted.

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A continuing focus in laboratory animal medicine has been to minimize the environmental factors that contribute to physiologic alterations within the research population.[11] Likewise, the microenvironment of laboratory mice has a profound effect on both the health and welfare of the animals as well as on the validity and reproducibility of scientific data.[28] The health effects of laboratory rodents, as a result of increased ammonia concentrations, has been described in various studies.[3,4,14,15,29,32,34] Likewise, the effects of various bedding substrates on intracage ammonia concentrations within IVC and static caging has been evaluated.[11,14,15,18,22,26,27,30,34] To our knowledge, however, no published studies have assessed the effects of various diet forms on intracage ammonia concentrations.

When designing an experiment that involves the use of laboratory animals, it is important to evaluate the physical form of the diet to minimize confounding influences and experimental variations.[36] In addition, reporting the physical form of a diet used in an experiment permits repeatability and the comparison of results with those of other studies.[36] Increases in cage soilage and the aroma of ammonia upon entry into the animal housing rooms of laboratory mice fed a pelleted laboratory animal diet have been reported anecdotally. In addition, cost-effectiveness can become a long-term concern, especially with large cohorts of animals. For example, the cost of the feed, feed disappearance, and the need to top off food in feed hoppers can potentially lead to increases in labor and per-diem costs, depending on the form of diet used.

Diets for laboratory animals are available in different types of physical forms, with pelleted diets being the most common.[23] This diet is typically manufactured by injecting steam into the mixture of ground ingredients, which then is forced through a die.[23] The shape of the pellet is determined by both the size and shape of the holes in the die, whereas the length is controlled by rotating blades; the diet is then allowed to dry until it is completely firm.[23] Advantages to using pelleted diets include their ease of handling, storage, and usage; reduction of dust in animal facilities; and compared with powdered or meal-form diets, have the tendency to reduce wastage. One noteworthy disadvantage of pelleted diets lies in the difficulty of altering or adding test compounds, once the diet has been manufactured.[23]

Another diet form is an extruded diet, which is similar to the pelleted diet. As with the pelleted diet, steam is injected into the ground meal, however, with the extruded diet, pressure and a higher temperature are used to force the meal through a die.[23] In addition, extruded diets are not as dense as pelleted diets and are typically preferred by some large animal species.[23] However, extruded diets are used less often than pelleted diets for laboratory rodents, one reason being the higher price per unit.[23] Furthermore, due to the formulation and decreased density of the extruded diet, extruded pellets break into chunks instead of the powdered fines observed with pelleted diets. The powdered fines that accumulate at the cage bottom presumably increase cage soilage, consequently increasing the frequency of cage change-outs and ultimately labor costs. Even though extruded diets cost more than pelleted diets, some institutions may prefer to feed their rodents extruded diet to decrease in cage soilage.

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Given these anecdotal observations, we hypothesized that, compared with mice that consumed a pelleted diet, those fed an extruded diet would maintain cages with less cage soilage, ultimately resulting in lower intracage ammonia concentrations. In addition, we wanted to evaluate the effects of these diet forms on feed disappearance, cage weight, and body weight. Furthermore, sex is an essential biologic variable to consider when designing an experiment, and its consideration in experimental design allows for transparency in reproducibility, including the ability to acknowledge any possible influences of sex on experimental outcomes in preclinical research.[6] We therefore evaluated whether any effects of diet form were influenced by the sex or strain or stock of the mice.

Materials and Methods

Animals. Inbred mice (age, 4 wk; athymic nude [Hsd:Athymic Nude-*Foxn1*^{nu}]; n = 60 male [average weight, 25.3 g], 60 female [average weight, 20.0 g]; C57BL/6 [C57BL/6NHsd]; n = 60 male [average weight, 20.6 g], 60 female [17.3 g]) and 4-wk-old outbred mice (ICR [Hsd:ICR(CD-1)]; 60 male [average weight, 36.0 g], 60 female [average weight, 28.0 g]) were obtained from a commercial vendor (Envigo, Indianapolis, IN). According to health surveillance programs, the mice were free of common adventitious agents as published on the vendor's monthly health reports.[10] Environmental conditions in the colony room were 70 to 74 °F (21 to 23 °C), with relative humidity of 40% to 55%, 20 air changes hourly, and a 12:12-h light:dark cycle. Mice were housed in IVC (model no. 1285, Tecniplast, Buggiatate, Italy) on racks (126 cages per rack, model 2L126MC36QLMDT, Tecniplast; 70 air changes per hour [Smart Flow Air Handling System, model BOX110SFMD, Tecniplast]). The cages contained autoclaved corncob bedding (1/4-in. Bed-o'Cobs; Andersons Lab Bedding Products, Maumee, OH), dispensed by an automatic bedding dispenser, and an autoclaved paper pack (Enviropak, Shepherd Specialty Papers, Watertown, TN) to serve as enrichment. This individually ventilated system recirculated room air, provided by the supply module, that allowed HEPA-filtered air to enter the system, and the exhaust module filtered spent air from the unit. In addition, mice had free access to reverse-osmosis–purified water through an automated watering system.

All research was conducted in compliance with the Animal Welfare Act[1] and other federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals*.[17] The protocol was approved by The University of Texas MD Anderson Cancer Center IACUC and was performed in an AAALAC-accredited facility.

Sanitation frequency. Cages containing mice were emptied of bedding and sanitized every 14 d, in compliance with the facility standards.

Diet types. We used 4 different irradiated diets in this study, representing 2 diets of the same composition but different forms from each of 2 manufacturers (no. 5053, PicoLab Rodent Diet 20 [P1], and no. 5R53, PicoLab Rodent Diet 20 Extruded [E1], PMI, St Louis, MO; no. 2918, Teklad Irradiated Global 18% Protein Rodent Diet [P2], and no. 2918X, Teklad Irradiated Global 18% Protein Extruded Rodent Diet [E2], (Envigo, Indianapolis, IN; Figure 1). Cages (n = per diet; 5 single-sex mice per cage) were randomly assigned for the mice to receive an allocated amount of 1 of the 4 irradiated diets. Each diet was measured individually, according to the weight of the feed necessary to completely fill the feed hopper. This weight was referred to as the standard weight and was consistently used throughout the study for each specified diet.

Diet	5053	5R53	2918	2918X
Treatment ID	P1	E1	P2	E2
Protein, %	21	21	18.6	18.6
Fat, %	5	5	6	6
ME, kcal/g	3	3	3.1	3.1
Form	Pellet	Extruded	Pellet	Extruded

Figure 1. Summary of diet compositions and forms.

Experimental design. We conducted a 14-d study to evaluate the effects of pelleted compared with extruded diets on intracage ammonia concentrations, feed disappearance, cage weight, body weight, and cage soilage. Prior to the onset of the study, all mice received the inhouse standard diet (P1) and were not manipulated, to facilitate acclimation to their new environment. After 3 d of acclimation, mice were each tattooed on the tail with a unique identification number, and baseline body weight measurements were obtained daily over 3 d. Mice were then placed on the diet specific to their treatment group and allowed an additional 2-wk acclimation period. The study began (day 0) when the mice were 6 wk old, and all animals were monitored daily for any signs of morbidity or mortality.

On days 0, 7, and 13, intracage ammonia levels were monitored for every cage. An ammonia detection tube (model CH20501, Ammonia 5/a, Draeger, Houston, TX) attached to a handheld gas analyzer pump (Accuro, Draeger) was inserted through the water port on the front of the cage, approximately 1 in. above the level of bedding, to detect intracage ammonia levels. The handheld pump was squeezed and released. The chemical preparation within the tube then reacted with the ammonia gas by changing color according to the ammonia concentration. Because cages did not have to be opened, each measurement took approximately 10 s only. In addition, ammonia concentrations were measured whenever feed was added to the feed hopper, in which case, ammonia was measured prior to opening the cage.

On days 0 and 14, each mouse and cage (including the cage bottom, bedding, and the enrichment) was weighed individually. Although the amount of bedding was added to each cage by using an automatic bedding dispenser and therefore was constant, each cage was weighed at the beginning and end of the study to account for any variabilities that might occur, but weight did not differ between the cages prior to the start of the study. Whenever the feed hopper was low, the remaining feed and additional feed were combined and then weighed so that the sum of the 2 weights was equivalent to the initial standard weight for each specified diet. At the completion of the study, the remaining feed in the feed hopper was weighed and removed, and the mice were returned to the inhouse standard diet (P1). Feed disappearance was calculated as the difference between the weight of the feed added and the feed remaining, divided by the number of animals per cage.[36]

Subjective scoring of cages. A single observer assessed the housing environment, at the cage level, throughout the 14-d study and independently scored all of the cages at 6 defined evaluation points (days 0, 3, 6, 9, 11, and 14). A modified Likert scoring scale was developed, and observations were assigned a value between 0 and 4 (Figure 2), according to the degree of soilage within the cage. Cage soilage reflected the amount of gross fecal and urine soiling inside the cage as well as any feed that fell into the cage bottom. These data were used to construct a graph, to compare the degree of cage soilage among cages according to the diet the mice consumed.

Statistical analyses. Data were evaluated by using ANOVA as a 4×3×2 factorial arrangement of treatments involving diet

Score	Description
0	Fresh cage; no urine or feces
1	Clean; little to no urine or feces; no saturation; cage change not needed. Condition: great.
2	Slightly soiled; little urine or feces; 25% saturation; cage change not needed. Condition: good.
3	Soiled; moderate urine and feces; 50% saturation; cage change needed. Condition: poor.
4	Extremely soiled; copious urine and feces; 75% saturation; cage change needed. Condition: very poor.

Figure 2. Cage scoring system and description of cage conditions

(E2, P2, E1, and P1), strain or stock of mouse (C57BL6, ICR, and nude), and sex (female and male) within a completely randomized design. Cage was the experimental unit. Initial and final body weights, feed disappearance, and cage weight were analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute, Cary, NC). The model included diet, strain, sex, and their interactions as fixed effects and the random effect of the cage within the diet. Least-squares means were compared by using the PDIFF option of SAS when protected by a significant ($P < 0.05$) treatment effect.

Comparisons of ammonia concentration, feed disappearance, cage soilage, cage weight, and body weight were analyzed by using the MIXED procedure of SAS for repeated measures.[20] The model included diet, strain or stock, sex, day, and all interactions. In MIXED models, the SEM depends on both σ^2_e and σ^2_b. Thus, in general, SEM in MIXED models account for 2 variance components. In our case, we are accounting for within- and between-cage variability. Using the Akaike Information Criterion and Schwarz Bayesian Criterion, we chose the most appropriate covariance structure from unstructured, compound symmetric, spatial power, and antedependence structures.[21] The Kenward–Rogers approximation was used for the calculation of the degrees of freedom of the pooled error term. The random effect of the cage within each diet (specified in the Subject statement) accounted for the correlation among repeated observations on the same cage. When diet×strain or stock×day, diet×sex×day, or diet×strain or stock×sex×day was significant ($P < 0.05$), means separations were evaluated on each day by using the PDIFF function of SAS.

Results

Body weight. Body weight demonstrated a 2-way interaction involving strain or stock and sex ($P < 0.0001$). By the end of the study, within each strain or stock, male mice weighed more ($P < 0.0001$) than females of the same strain or stock (Figure 3). Body weight (mean ± SEM; $n = 60$) at the end of the study was 21.3 ± 0.3 g for female C57BL/6, 24.3 ± 0.3 g for male C57BL/6, 35.5 ± 0.3 g for female ICR, 44.1 ± 0.3 g for male ICR, 22.8 ± 0.3 g for female nude, and 28.8 ± 0.3 g for male nude mice.

Feed disappearance. Feed disappearance was influenced by diet ($P < 0.0001$) and by strain or stock ($P < 0.0001$). Regarding diet, the greatest feed disappearance (75.8 ± 1.9 g) was seen with mice that consumed P1 (Figure 4). Among strains or stock, ICR mice had the greatest feed disappearance (72.9 ± 1.7 g) and C57BL/6 mice the least (47.5 ± 1.7 g; Figure 5). Feed disappearance differed significantly between each strain or stock ($P < 0.0001$ for all 3: C57BL/6×ICR, C57BL/6×nude, and ICR×nude).

Cage weight. A 3-way interaction involving strain or stock, sex, and diet occurred ($P = 0.0446$). The initial weight did not

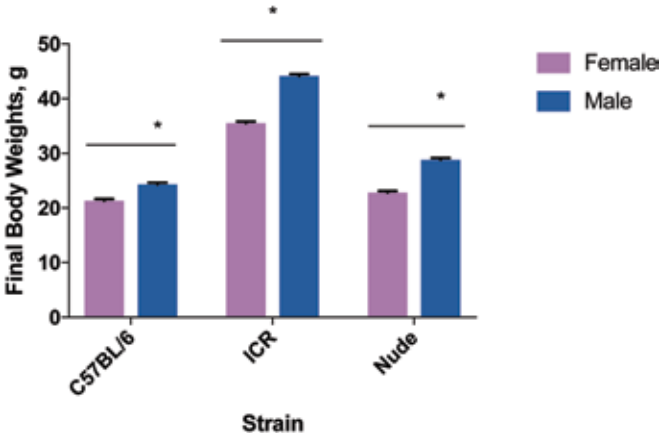

Figure 3. Effect of strain or stock and sex on final body weight (g, mean ± SEM; $n = 60$). Body weight was influenced ($P < 0.0001$) by a strain or stock×sex interaction. *, Values differ significantly ($P < 0.05$) between females and males of the same strain or stock.

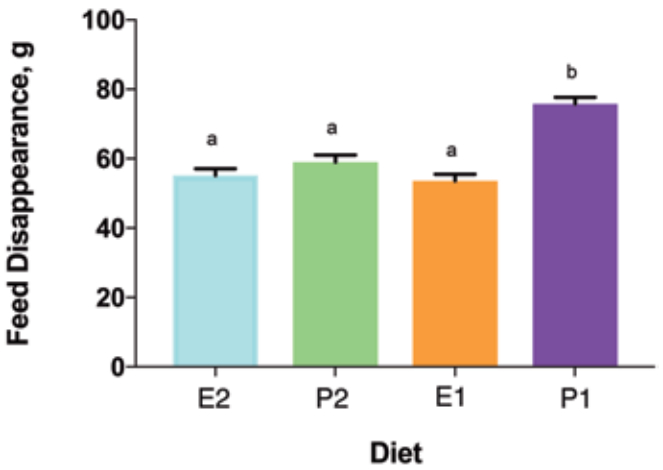

Figure 4. Effect of diet (E2, P2, E1, and P1) on feed disappearance (g, mean ± SEM; $n = 18$). Feed disappearance was influenced ($P < 0.0001$) by diet. Different letters indicate significantly ($P < 0.05$) different values.

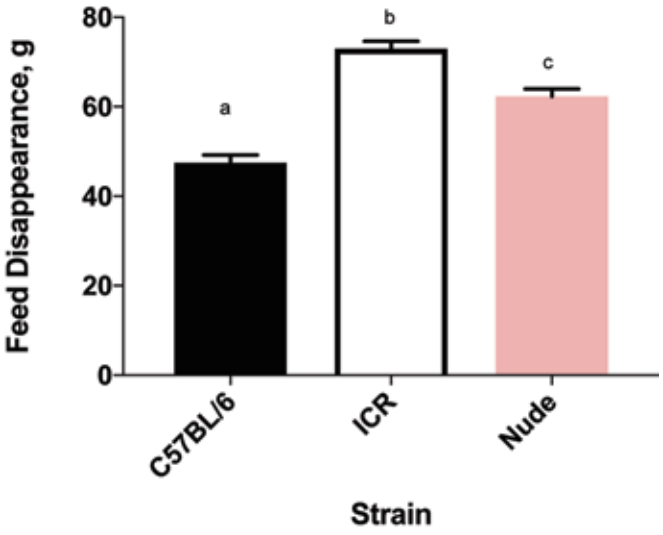

Figure 5. Effect of strain or stock on feed disappearance (g, mean ± SEM; $n = 24$). Feed disappearance was influenced ($P < 0.0001$) by strain or stock. Different letters indicate significantly ($P < 0.05$) different values.

differ among the fresh cages. The cages housing C57BL/6 female mice that consumed E1 were heavier ($P = 0.0013$) than those housing male C57BL/6 mice that consumed E1. No other differences were seen between male and female C57BL/6 mice that consumed any of the 3 remaining diets. When compared with other C57BL/6 male mice, those that consumed P1 had the heaviest cages at study end. Similarly, the cages of female C57BL/6 mice that consumed P1 were heavier than those of female C57BL/6 mice that consumed either E2 or P2 ($P = 0.0377$ and $P = 0.0534$, respectively); but no differences were seen among female C57BL/6 mice that consumed either E1 or P1 ($P = 0.1572$; Figure 6 A). Female ICR mice given P1 had heavier ($P = 0.0089$) cages than male ICR mice on P1. Overall, both male and female ICR mice that consumed P1 generated greater ($P < 0.05$) cage weights than did male and female ICR mice that had any of the 3 remaining diets (Figure 6 B). Nude: Cage weights were greater ($P < 0.05$) for both male and female nude mice that consumed P1 than any of the 3 remaining diets (Figure 6 C). Table 1 contains a summary of the least-square means for cage weight.

Intracage ammonia concentration. A 3-way interaction involving strain or stock, diet, and day was present ($P = 0.0004$). For all mice, intracage ammonia increased numerically relative to day 0 as the study progressed. Ammonia concentrations in cages that housed C57BL/6 mice did not differ on days 7 and 13 for any of the dietary groups (Table 2). In cages that housed ICR mice, ammonia concentrations differed between P2 and E1, such that cages with mice that consumed P2 had higher ($P = 0.0496$) ammonia concentrations than those that consumed E1. On day 7, the cages of ICR mice that consumed P2 had the lowest ammonia concentrations (P2 compared with E2, $P = 0.0348$; P2 compared with E1, $P = 0.0007$; and P2 compared with P1, $P = 0.0107$). On day 13, cages of ICR mice that consumed P2 had lower ($P \leq 0.05$) ammonia concentrations than did cages of ICR animals given E2. No differences were seen in the cages of ICR mice that consumed either E1 or P1 (Table 2). Cages that housed nude mice that consumed P2 showed increased ammonia concentrations on day 0 compared with those given E2 ($P = 0.0329$) or E1 ($P = 0.0004$). On day 7, nude mice that consumed P2 had lower intracage ammonia levels, compared with cages of nude mice that consumed any of the 3 remaining diets (P2 compared with E2, $P = 0.0197$; P2 compared with E1, $P = 0.0197$; P2 compared with P1: $P < 0.0001$). The greatest ammonia concentrations occurred in the cages of nude mice given P1, compared with those cages of nude mice on any of the 3 remaining diets (P1 compared with E2: $P = 0.0057$, P2: $P < 0.0001$, and E1: $P = 0.0057$). In addition, the cages of nude mice that consumed E1 had greater ammonia concentrations than those of nude mice provided either E2 or P2 (Table 2).

Cage score. Cage score was influenced by strain or stock×sex ($P = 0.0389$), sex×diet ($P = 0.0032$), strain or stock×day ($P < 0.0001$), and diet×day ($P = 0.0004$) interactions. C57BL/6 and ICR mice had higher ($P \leq 0.05$) cage scores than did nude mice, with nude males having higher scores than females ($P = 0.0006$); no differences were seen between male and female C57BL/6 or ICR mice (Figure 7). Male mice that consumed E2 had higher ($P < 0.0001$) cage scores than female mice that consumed the same diet; no differences occurred between male and female mice that consumed any of the 3 remaining diets (Figure 8). For all strains and stock, cage scores increased at each measurement compared with day 0. However, cage scores were similar between days 11 and 14 for C57BL/6 ($P = 0.4025$) and nude ($P = 0.2096$) mice. For mice that consumed E2, similar scores occurred days 3 and 6 ($P = 0.0539$) and days

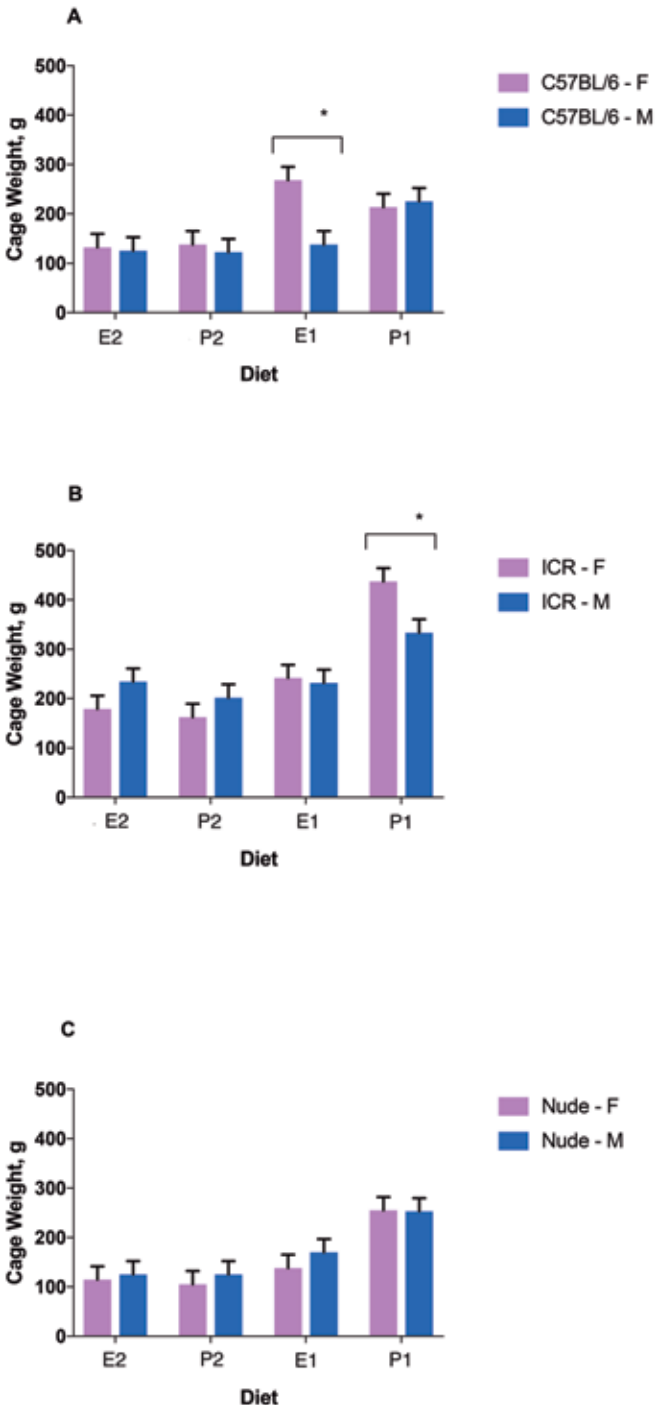

Figure 6. Effect of strain or stock, sex, and diet (E2, P2, E1, and P1) on cage weight (g, mean ± SEM; $n = 3$). (A) Female and male C57BL/6 mice, (B) female and male ICR mice, and (C) female and male nude mice. Cage weight was influenced ($P = 0.0446$) by a strain or stock×sex×diet interaction. *, Values differ significantly ($P < 0.05$) between females and males fed the same diet.

11 and 14 ($P = 0.1476$). For mice that consumed P2, cage scores increased with each time point from day 0 to day 11 but were similar between days 11 and 14 ($P = 0.0539$). Similarly, mice that consumed E1 had increases in cage scores until day 11, but no differences were seen between days 11 and 14 ($P = 0.3338$). No differences in cage score occurred for mice that consumed P1 after day 9 (days 9 and 11, $P = 0.2641$; days 9 and 14, $P = 0.0959$; and days 11 and 14: $P = 0.3338$).

Table 1. Least-square means of final cage weights (g; mean ± SEM; $n = 3$) of IVC housing C57BL/6, ICR, or nude mice that consumed the E1, P1, E2, or P2 diet

	E1		P1		E2		P2	
	F	M	F	M	F	M	F	M
C57BL/6	268.3 ± 26.9	138.3 ± 26.9[a]	213.7 ± 26.9	225.3 ± 26.9	132.3 ± 26.9	125.7 ± 26.9	138.3 ± 26.9	122.7 ± 26.9
ICR	241.7 ± 26.9	231.7 ± 26.9	437.3 ± 26.9	333.7 ± 26.9[a]	179.3 ± 26.9	234.3 ± 26.9	162.7 ± 26.9	202.0 ± 26.9
Nude	138.3 ± 26.9	170.0 ± 26.9	255.0 ± 26.9	253.0 ± 26.9	114.7 ± 26.9	125.3 ± 26.9	105.3 ± 26.9	125.3 ± 26.9

Cage weight was influenced ($P = 0.0446$) by a strain or stock×sex×diet interaction.
[a]Values differed significantly ($P < 0.05$) for females compared with males fed the same diet.

Table 2. Least-square means of ammonia concentrations (ppm; mean ± SEM; $n = 6$) on day 0, 7, or 13 of IVC housing C57BL/6, ICR, or nude mice that consumed the E1, P1, E2, or P2 diet

	E1			P1			E2			P2		
Day	B6	ICR	Nude	B6	ICR	Nude	B6	ICR	Nude	B6	ICR	Nude
0	13.3 ± 3.2	12.5 ± 3.2[a]	5.0 ± 3.2	6.7 ± 3.2	20.8 ± 3.2	14.2 ± 3.2	10.8 ± 3.2	15.8 ± 3.2	12.50 ± 3.2[a]	8.33 ± 3.2	21.7 ± 3.2[a]	22.5 ± 3.2[a]
7	66.7 ± 12.2	125.0 ± 12.2	50.0 ± 12.2	50.0 ± 12.2	108.3 ± 12.2	100.0 ± 12.2[a]	45.8 ± 12.2	100.0 ± 12.2	50.00 ± 12.2	54.2 ± 12.2	62.5 ± 12.2[a]	8.3 ± 12.2[a]
13	66.7 ± 13.2	125.0 ± 13.2	91.7 ± 13.2[a]	58.3 ± 13.2	125.0 ± 13.2	137.5 ± 13.2[a]	58.3 ± 13.2	141.7 ± 13.2[a]	51.67 ± 13.2	66.7 ± 13.2	95.8 ± 13.2[a]	52.5 ± 13.2

Ammonia was influenced ($P = 0.0004$) by a strain or stock×diet×day interaction.
[a]Value differs significantly ($P < 0.05$) from others for that day.

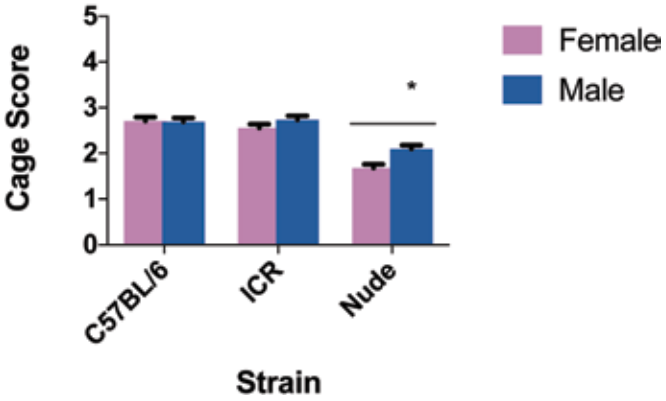

Figure 7. Effect of strain or stock and sex on cage score (mean ± SEM; $n = 12$). Cage score was influenced ($P = 0.0389$) by a strain or stock×sex interaction. *, Values differ significantly ($P < 0.05$) between females and males of the same strain or stock.

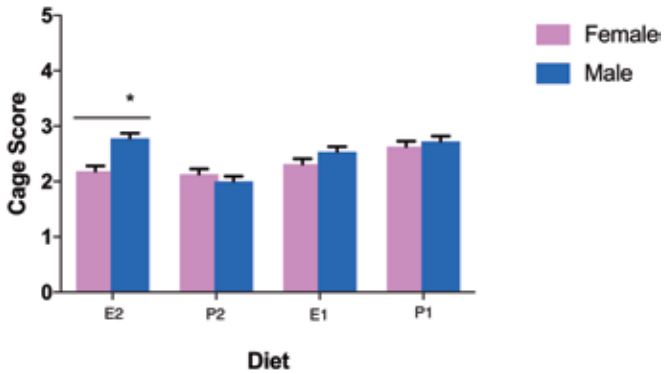

Figure 8. Effect of sex and diet (E2, P2, E1, and P1) on cage score (mean ± SEM; $n = 12$). Cage score was influenced ($P = 0.0032$) by a sex×diet interaction. *, Values differ significantly ($P < 0.05$) between females and males of the same strain or stock.

Discussion

Many variables must be considered when assessing intracage ammonia concentrations, including sex, strain or stock, the health status of the animals, bedding, environment, the frequency of cage changes, and housing density. To our knowledge, no previous studies have assessed the effect of diet form on intracage ammonia concentrations.[13,35] In the current study, we assessed the effects of extruded and pelleted diets on feed disappearance, body weight, and cage score, which was based on the degree of cage soilage. We also evaluated whether these effects were influenced by the strain or stock or sex of the mice. This study reveals that the physical form of the diet does indeed affect cage weight, feed disappearance, cage soilage, and intracage ammonia concentration (Tables 3 and 4). We also evaluated additional environmental parameters, including intracage temperature, humidity, and CO_2, during this study but will publish those findings elsewhere.

Diet form did not affect the final body weights of any of the strains or stock of mice. Instead, the body weights were influenced by the strain or stock and sex of the mice. At the completion of this study, the mice were approximately 8 wk old. According to the vendor's growth chart for ICR mice, 8-wk-old females have a mean body weight of 33.3 g, and the mean weight of males is 41.5 g;[9] 8-wk-old athymic nude female mice obtain a mean body weight of 21.0 g and males a mean of are 26.4 g.[7] In addition, 8-wk-old C57BL/6 female mice weigh 18.4 g on average and males are a mean of 23.7 g.[8] This information was consistent with the body weights that we saw in our 8-wk-old mice at study completion. Overall, ICR mice typically are heavier than nude or C57BL/6 mice; C57BL/6 mice typically weigh less than ICR and nude mice, with male mice weighing more than females.

The difference in the weight of the feed added and the feed remaining, divided by the number of mice per cage, was calculated as feed disappearance.[16] Feed disappearance, however, does not account for the feed that reaches the bottom of the cage

Table 3. Summary of strain or stock differences and the effects of diet on several microenvironmental parameters

	C57BL/6	ICR	Nude
Final body weight (g)	Lowest final body weights; male mice weighed more than female mice.	Greatest final body weights; male mice weighed more than female mice.	Male mice weighed more than female mice.
Feed disappearance (g)	Least amount of feed disappearance.	Greatest amount of feed disappearance.	
Final cage weight (g)	Of mice that consumed E1, females had greater cage weights than males.	Of mice that consumed P1, females had greater cage weights than males; P1 was the most consumed diet among this strain or stock	No significant differences seen between males and females; P1 was the most consumed diet among this strain.
Ammonia concentration (ppm)	Ammonia concentrations plateaued by day 7; no statistical differences seen between or within the diets by day 13.	By day 13 (end of study), highest concentrations were seen with E2 and the lowest were with P2.	By day 13 (end of study), highest concentrations were seen with P1, and the lowest levels were among E2 and P2.
Cage score (soilage)	No differences in degree of cage soilage between males and females. Overall, cage scores increased with time.	Overall, cage scores increased with time.	Males had greater cage scores than females. Overall, cage scores increased with time.

Table 4. Summary of diet-associated effects on several microenvironmental parameters

	E2	P2	E1	P1
Final body weight (g)	No effect	No effect	No effect	No effect
Feed disappearance (g)	No significant difference between E2, P2, and E1	No significant difference between E2, P2, and E1	No significant difference between E2, P2, and E1	Showed greatest feed disappearance
Final cage weights (g)			Both B6 and ICR females fed E1 had heavier cage weights than counterpart males	Greatest consumption by nude and ICR mice.
Ammonia concentration (ppm)	Lowest day 13 levels in nude mice; highest day 13 levels in ICR mice; day 13 levels did not differ among diets in B6 mice.	Lowest day 13 levels in ICR mice.		Highest day 13 levels in nude mice
Cage score (soilage)	Male mice had greater cage scores than female mice; overall, cage scores increased with time.	No significant differences between male and female mice; overall, cage scores increased with time.	No significant differences between male and female mice; overall, cage scores increased with time.	No significant differences seen between male and female mice; overall, cage scores increased with time.

and ultimately becomes mixed with the bedding and excreta. This quantity of feed is considered wastage and is accounted for in the difference in cage weights from the beginning to end of the study.

Throughout the study, we noted the accumulation of powdered fines in the cage bottoms of mice that consumed the P1 diet—but not any of the other diets; these fines would explain the high feed disappearance for mice that consumed P1. In addition, mice consume 3 to 5 g of feed daily after weaning and maintain this intake throughout life.[12] However, some of the larger strains or stocks, such as ICR mice, may eat as much as 8 g daily per animal.[19] This increase in feed intake and the large sizes of ICR mice explain the increased amounts of feed disappearance seen with this strain. Likewise, the fact that C57BL/6 mice typically weigh less than ICR and nude mice might mean that C57BL/6 mice consume less feed. This difference can ultimately explain the low amounts of feed disappearance seen with the C57BL/6 mice in the current study.

The increased cage weights seen with the P1 diet are consistent with the accumulation of powdered fines at the cage bottoms, as previously mentioned. In addition, increased cage weights might indicate an increased amount of soiled bedding, thus increasing the frequency of cage changes and associated labor costs. Furthermore, increased frequency of cage changes

can have negative effects on both the mice as well as the health of the personnel performing these tasks.[35] Frequent cage changing can increase stress in mice, potentially resulting in increased aggression and stereotypic behaviors in mice.[35] In addition, staff members experience increased exposure to allergens, dust, and ammonia; these effects typically are reduced through wearing personal protective equipment, such as face masks, and using laminar flow hoods or biosafety cabinets.[35]

The limits of a person's exposure to any toxic airborne substance are used to determine the threshold limit value to which a person can be exposed for 8 h daily, 5 d a week, without any harmful effects.[14,25] Ammonia is irritating to human skin, eyes, and lungs, and the current exposure limit set by the Occupational Health and Safety Administration and the American Conference of Governmental Industrial Hygienists is 50 ppm maximal exposure, or 25 ppm averaged over an 8-h work day.[2,24] Similarly, the National Institute for Occupational Safety and Health Standards has established recommended exposure limits that are time-weighted average concentrations for a maximal 10-h workday during a 40-h workweek;[5] for ammonia, the recommended time-weighted average is 25 ppm.[5] Without absolute ammonia exposure limits in rodents, the guideline for maximal ammonia exposure of rodents is often 50 ppm. Interestingly, some studies have shown no histologic differences among rodents exposed to various ranges of ammonia concentrations. For example, histology of the nasal passages did not differ even though the mice were exposed to a variety of cage-changing frequencies, ventilation rates, and ammonia concentrations, ranging from less than 25 ppm to more than 100 ppm.[26,30] In contrast, some studies have shown a promotion of the growth of infective agents, such as *Mycoplasma pulmonis*, in the respiratory tract of rats exposed to ammonia concentrations of less than 25 ppm[4,27] as well as tracheal epithelial inflammatory changes with an ammonia concentration of 200 ppm.[14] In addition, one review provides a thorough summary of the effects of exposure to ammonia on various species,[31] including mortality in rats,[3] depressed immune responses in guinea pigs,[32] and decreased concentration-dependent wheel running in Long-Evans rats and Swiss mice, with the rats showing more of a decrease in activity than the mice.[33] In the current study, we recorded intracage ammonia levels as high as 200 ppm with no obvious clinical signs of respiratory effects, such as respiratory distress. Although no histopathologic evaluations were performed in this study, it is important to emphasize the importance and potential effects that intracage ammonia concentrations can have on laboratory rodents.

The longer the soilage was left in the cage, we expected that the intracage ammonia concentrations would increase as well, similar to another study's findings.[35] However, this pattern was not the case with cages housing C57BL/6 mice. Instead, across all 4 diets, these cages actually showed no significant increase after day 7 of the study. In addition, cages of mice that consumed P1 had increasing concentrations of ammonia, particularly in the cages that housed the nude mice. In fact, intracage ammonia concentrations were the highest on both days 7 and 13 for nude mice that consumed P1, compared with all the other diets. Why this result occurred is unclear, but it does reveal that, at least in nude mice, consumption of P1 leads to increased levels of ammonia. We saw a similar trend in the cages housing nude mice that consumed E1. This result may provide justification for increasing the frequency of cage changes for nude mice that consume either of the diets from vendor 1.

Furthermore, on day 7, nude mice that consumed P2 had a decrease in intracage ammonia levels, but these levels increased again by day 13. This decrease in ammonia levels was only seen with the nude mice on day 7 and most likely was due to a technical error caused by the handheld gas analyzer pump. All 6 cages of nude mice that consumed P2 were affected by this technical error, and none of these cages had been opened prior to this reading. It is also important to note that, by the end of the study, the lowest intracage ammonia levels occurred in the cages of nude mice that consumed either the P2 or E2 diet. Likewise, cages with ICR mice that were fed P2 maintained the lowest levels of intracage ammonia concentrations on days 7 and 13, in comparison to ICR mice fed the counterpart diet, E2. The reason for this effect is unclear, but this outcome indicates that, at least in ICR mice, low ammonia concentrations are seen when they consume P2. Overall, by the end of the 14-d study, low ammonia concentrations were seen for 2 of the strains that consumed P2. Although this information disproves our hypothesis, it still confirms that multiple variables must be considered when assessing the effects of diet on intracage ammonia concentrations. In addition, these data provide validating information for institutions to include when evaluating alternative diets for their mice. Furthermore, we found it noteworthy that the P2 diet was the only diet that did not need to be topped off within any of the cages, thus making it more cost-effective as well. It merits mentioning, that a limitation to this study is that cages that needed feed to be added had to be opened to do so, thus allowing for the dissipation of gaseous ammonia from the cages. Lastly, although previous studies evaluating various bedding types have reported higher ammonia concentrations with male mice than female mice, we noted no such sex-associated differences in our current study.[13]

As previously mentioned, throughout this study, cages housing mice that consumed the P1 diet had a large accumulation of powdered fines at the bottom of the cages. These powdered fines add to the normal cage soilage (that is, feces, urine) and allow fluids such as water and urine to be further absorbed into both the bedding and the feed, leading to additional moisture accumulation within the cage. Particle size of bedding may play an important role in desiccating fecal pellets and thus reduce ammonia production.[25] Large particles, having a larger exposure area, likely would result in a faster rate of moisture evaporation.[25] Consequently, poor absorption by bedding might thus be a contributing factor toward higher ammonia levels.[25] The same implication can be applied to diet form. The particle size of the feed that falls to the cage floor may actually play an important role in desiccating fecal pellets and thus reduce ammonia production. If the feed on the cage floor absorbs poorly, this characteristic might ultimately be a contributing factor toward increased ammonia concentrations. However, we did not evaluate the absorption properties of these particular diets in the current study, and this attribute should be investigated in future studies.

In this study, the degree of cage soilage was influenced by several variables. Overall, the degree of cage soilage was the greatest for both the C57BL/6 and ICR mice and the lowest in the cages housing nude mice. With ICR mice being the largest of the 3 mouse types we evaluated and given that they had the greatest feed disappearance, their increased cage soilage and thus higher cage scores was no surprise. Regarding the C57BL/6 mice, perhaps the rate of metabolism and potential for grinding, although not evaluated in our study, are responsible for the increase in cage soilage compared with that of nude mice. In addition, nude female mice had the lowest degree of cage soilage compared with male nude mice and with both ICR and C57BL females and males. Furthermore, male mice that

consumed E2 had higher cage scores than female mice that consumed the same diet. Although statistical differences were not seen between males and females for the 3 remaining diets, male mice that consumed P2 obtained lower cage scores. As previously mentioned, the absorption properties of these diets were not evaluated in this study; however, the lower cage scores seen with male mice that consumed P2 suggests that the other 3 diets have poorer absorption.

As the days progressed, the soilage likewise continued to increase with each of the strains or stock, with the exception of both C57BL/6 and nude mice, which appeared to reach a plateau at day 11. These findings further demonstrate that both C57BL/6 and ICR mice maintained the most soiled cages throughout the study. These results may provide justification for an increase in cage change frequency, by day 11 for C57BL/6 and ICR, for institutions that change cages every 14 d. Although differences were not always statistically significant, mice that consumed P2 maintained the lowest level of cage soilage throughout the entire study. This finding is consistent with the overall lower intracage ammonia concentrations seen with both nude and ICR mice that consumed P2 as well as with the fact that this diet did not have to be topped off during the study, indicating decreased waste with the P2 diet. The cage score and ammonia concentration data further support the idea that the P2 diet provides greater absorption than the other diets. This conclusion again disproves our hypothesis, given that P2 is a pelleted diet; however, cages of mice fed the P2 diet clearly would need fewer 'spot changes' between scheduled cage change-out periods, thus ultimately lowering labor costs. In addition to the lower intracage ammonia concentrations, the decreased cage soilage would contribute to the increased cost-effectiveness of the P2 diet.

Our results show clear differences between pelleted and extruded diets in regard to intracage ammonia concentrations, feed disappearance, cage weight, and cage soilage. These findings are immediately applicable in laboratory animal facilities using these diet forms and demonstrate the importance of considering the physical form of the diet in the experimental design.

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