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Effects of Housing Density and Cage Floor Space on C57BL/6J Mice

Abigail L. Smith, PhD,[1,*,†] Sarah L. Mabus,[1] Jason D. Stockwell, PhD,[1,‡] and Cameron Muir, PhD[2]

The *Guide for the Care and Use of Laboratory Animals* (the *Guide*) is widely accepted as the housing standard by most Institutional Animal Care and Use Committees. The recommendations are based on best professional judgment rather than experimental data. Current efforts are directed toward replacing these guidelines with data-driven, species-appropriate standards. Our studies were undertaken to determine the optimum housing density for C57BL/6J mice, the most commonly used inbred mouse strain. Four-week-old mice were housed for 8 weeks at four densities (the recommended ~12 in² [ca. 77.4 cm²]/mouse down to 5.6 in² [ca. 36.1 cm²]/mouse) in three cage types with various amounts of floor space. Housing density did not affect a variety of physiologic parameters but did affect certain micro-environmental parameters, although these remained within accepted ranges. A second study was undertaken housing C57BL/6J mice with as little as 3.2 in²/mouse (ca. 20.6 cm²). The major effect was elevated ammonia concentrations that exceeded limits acceptable in the workplace at increased housing densities; however, the nasal passages and eyeballs of the mice remained microscopically normal. On the basis of these results, we conclude that C57BL/6J mice as large as 29 g may be housed with 5.6 in² of floor space per mouse. This area is approximately half the floor space recommended in the *Guide*. The role of the *Guide* is to ensure that laboratory animals are well treated and housed in a species-appropriate manner. Our data suggest that current policies could be altered in order to provide the optimal habitation conditions matched to this species' social needs.

The *Guide for the Care and Use of Laboratory Animals* (10) specifies floor space requirements for laboratory mice of different weights. All cages must be at least 5 in. (ca. 12.7 cm) high. Floor space requirements (per mouse) are designated as at least 6 in^2 (ca. 38.7 cm^2) for mice less than 10 g, 8 in^2 (ca. 51.6 cm^2) for mice up to 15 g, 12 in^2 for mice up to 25 g, and more than 15 in^2 (ca. 96.8 cm^2) for mice weighing more than 25 g. The few peer-reviewed publications that address the floor space needs of laboratory mice suggest that mice can be housed at densities higher than those recommended in the *Guide* (10) and that mice housed at higher densities are healthier and less aggressive than mice housed at lower densities (6, 13, 27). We recognize that laboratory mice are quite removed from their wild progenitors; however, the husbandry procedures we impose on laboratory mice may be based more on the aesthetic impact on caretakers and investigators rather than what is really best for the mice.

Rodent population densities have been shown to alter a number of normal and experimental parameters including food consumption (11), tumor growth (1), unique ovarian lesion development (4), testosterone secretion (19), testis weight (22), suppressed thermogenic capacity of brown adipose tissue due to "social thermoregulation (huddling)" (9), cholesterol levels and fatty lesions in the aortas of female mice (12), development of autoimmunity (8), and plasma corticosterone and peripheral lymphocyte populations (20). Table 1 summarizes the results from several studies that examined a variety of physiologic parameters among mice provided with different amounts of floor space. In general, provision of less floor space either had no effect or was beneficial, resulting in reduced mortality, reduced aggression, and enhanced immune responses. Fullwood and co-workers (6) concluded that provision of less floor space per mouse was not detrimental, despite elevated corticosterone levels and increased adrenal gland weight, because both T cell mitogen and natural killer cell responses were enhanced.

The existing literature does not collectively provide sufficient insight regarding space allocation requirements of mice for optimal physiologic parameters that are indicative of well-being. The studies reported here were designed to reveal how floor space, in the context of cage size versus population density, might influence several parameters in young adult C57BL/6J (B6) male and female mice. The indices we studied were survival, aggressive behavior or injuries, body weight, food and water consumption, cage micro-environment (in-cage ammonia and CO_2 levels, temperature, and relative humidity), hair loss (a commonly observed characteristic in B6 mice, particularly young adult females), urinary testosterone concentrations, and microscopic evidence of ammonia damage to nasal passages and eyeballs. Other investigators (6, 13) have emphasized that floor space and group size can be confounding variables. In those studies, constant numbers of mice were housed in cages with variable floor space modified by partitions. Although we appreciate the merits of that study design, housing of laboratory rodents is dependent on the cage designs available in the commercial sector. For this reason, we have studied housing density in three readily available types

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[1]The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609, [2]Department of Psychology, Brock University, 500 Glenridge Avenue, St. Catherines, Ontario, Canada L2S 3A1.
[†]Present address: University of Pennsylvania, ULAR/Suite 220, Mail code 3512, 229 S. 40th Street, Philadelphia, Pennsylvania 19104.
[‡]Present address: USGS—Great Lakes Science Center, Lake Superior Biological Station, 2800 Lakeshore Drive, Ashland, Wisconsin 54806.
[*]Corresponding author.

Table 1. Published effects of providing laboratory mice with different amounts of floor space

Floor space per mouse	Mouse strain (sex)	Endpoint(s)	Result with less floor space	Reference
12.4 in^2, 19.4 in^2 (ca. 80.0 cm^2, 125.1 cm^2)	BALB/cAnNCrlBr (M)	Aggression	Reduced	27
5.0–20.0 in^2 (ca. 32.3–129.0 cm^2)	C57BL/6 (M)	Rate of weight gain Food, water consumption Immune function Corticosterone levels Adrenal weight Mortality	Unaffected Increased Enhanced Increased Increased Decreased	6
5.0–20.0 in^2 (ca. 32.3–129.0 cm^2)	BALB/cJ (M, F)	Rate of weight gain Food, water consumption Immune function Mortality	Unaffected or increased Unaffected Unaffected or enhanced Unaffected or reduced	13
6.5–52.0 in^2 (ca. 41.9–335.4 cm^2)	Multiple inbred (F)	Food waste Heat conservation (huddling) Metabolic rate	Reduced Increased Reduced	11
3.4–20.5 in^2 (ca. 21.9–132.2 cm^2)	OF1 (M)	Testosterone levels Response to acute noise stress Adrenal, testis weights Body weight	Unaffected Unaffected Unaffected Reduced	19
4.2–8.5 in^2 (ca. 27.1–54.8 cm^2)	BALB/c, MF1 (M, F)	Growth rate, adrenal weight	Unaffected	21

M, male; F, female.

of cages—two used extensively at The Jackson Laboratory (TJL; i.e., duplex and weaning cages) and the "shoebox" cages used at most other facilities. Populations of B6 mice were housed in each cage type (each having different amounts of floor space) at four different densities—one compatible with recommendations in the *Guide* and three higher densities. A second study was performed to determine whether recently weaned B6 male and female mice could be housed with even less floor space (down to 3.2 in^2 per mouse). We conclude that male and female B6 mice, between the ages of 4 and 12 weeks, can be housed with 5.6 in^2 of floor space per mouse without ill effect. This area is approximately half the floor space recommended in the *Guide* (10).

Materials and Methods

Mice. For each experiment, 4-week-old C57BL/6J male and female mice were obtained from JAX Research Systems (Bar Harbor, Maine). The primary 8-week study involved 538 mice of each sex, and 660 mice of each sex were used in the 4-week follow-up experiment. They were weighed and then housed for 8 weeks (primary study) or 4 weeks (follow-up study) in positively ventilated cage racks (Thoren Caging Systems, Inc., Hazleton, Pa.) and were provided ad libitum with 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 and acidified water (pH 2.8 to 3.1, monitored continuously). The ventilation rate was 45 air changes per hour, and the racks were vented out of the room. Cages were processed through a tunnel washer. Bedding consisted of autoclaved white pine shavings (Crobb Box Co., Ellsworth, Maine) and was changed weekly. The light cycle was 14 h light:10 h dark. All colonies at JAX Research Systems are regularly monitored for and are free of 17 viruses (ectromelia virus, Theiler's mouse encephalomyelitis virus, K virus, hantavirus, lactic dehydrogenase-elevating virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse parvovirus, mouse adenovirus, mouse cytomegalovirus, mouse hepatitis virus, mouse thymic virus, pneumonia virus of mice, polyoma virus, reovirus 3, murine rotavirus [EDIM], and Sendai virus), 17 bacterial species (including *Helicobacter* spp.), two *Mycoplasma* spp., external and intestinal parasites, and *Encephalitozoon cuniculi*. The Institutional Animal Care and Use Committee approved the reported experiments. Mice were euthanized with CO_2 gas at the end of the experiments.

Cage types. We used three polycarbonate cage types available from Thoren Caging Systems Inc. They were duplex cages (floor space per side = 51.7 in^2 [ca. 333.5 cm^2]), weaning cages (112.9 in^2 [ca. 728.4 cm^2]) and Thoren #1 "shoebox" cages (67.6 in^2 [ca. 436.1 cm^2]). The duplex and weaning cage fit into the same slots in a ventilated cage rack. Table 2 shows the floor space provided per mouse, the samples sizes (numbers of cages), and number of mice used for each density and cage type in the primary 8-week study. Table 3 shows the same information for the 4-week follow-up study.

Parameters measured and frequency. Injury, aggressive behavior, and survival were assessed daily during water bottle checks. Body weights were measured on the first day and, along with food and water consumption, at weekly intervals thereafter. Post-husbandry behavior was observed weekly immediately after mice were placed in clean cages. Cage micro-environment (see following section) was monitored weekly in the 8-week study, just prior to placing mice on fresh bedding, and twice weekly in the 4-week study. Mice were also inspected weekly for hair loss. Urine was collected at study initiation and bi-weekly (8-week study) or weekly (4-week study) thereafter and was frozen at –80°C until testosterone assays were performed. To assess ammonia toxicity, nasal passages and eyeballs from representative mice from each housing density were examined microscopically at the termination of the 4-week study.

Cage micro-environment monitoring. In the primary study, ammonia, carbon dioxide, temperature, and relative humidity (RH) were measured weekly with an Innova multi-gas analyzer (Innova AirTech Instruments A/S, Ballerup, Denmark) in cages occupied by male B6 mice. Males were chosen because we assumed that their values would be higher in most categories. However, in the 4-week study, we measured micro-environmental parameters in cages of both male and female mice twice weekly. Time-based differences were analyzed because the mice were growing, and their biomass and urine output changed over time. This growth could certainly affect in-cage ammonia levels

Table 2. Cage types, housing densities, and numbers of cages and C57BL/6J male or female mice studied at each density

Duplex cages (six cages per density level)			Shoebox cages (four cages per density level)			Weaning cages (four cages per density level)			
Floor space/ mouse	No. mice/ pen	Total no. mice at density level	Floor space/ mouse	No. mice/ cage	Total no. mice at density level	Floor space/ mouse	No. mice/ cage	Total no. mice at density level	Density code
12.9 in^2 (ca. 83.2 cm^2)[a]	4	24	11.2 in^2 (ca. 72.2 cm^2)[a]	5	20	12.5 in^2 (ca. 80.6 cm^2)[a]	9	36	1
8.6 in^2 (ca. 55.5 cm^2)	6	36	8.5 in^2 (ca. 54.8 cm^2)	8	32	8.7 in^2 (ca. 56.1 cm^2)	13	52	2
6.5 in^2 (ca. 41.9 cm^2)	8	48	6.8 in^2 (ca. 43.9 cm^2)	10	40	6.6 in^2 (ca. 42.6 cm^2)	17	68	3
5.7 in^2 (ca. 36.8 cm^2)	9	54	5.6 in^2 (ca. 36.1 cm^2)	12	48	5.6 in^2 (ca. 36.1 cm^2)	20	80	4
Total		162			140			236	

[a]Floor space/mouse is the *Guide* (14) recommendation for mice weighing up to 25 g, although some mice weighed more than 29 g at the termination of the 8-week study.

Table 3. Housing densities and numbers of C57BL/6J male or female mice studied at each density in weaning cages

Floor space/ mouse	No.mice/ cage	Total no. mice at density level	Density code
5.6 in^2 (ca. 36.1 cm^2)	20	120	4
4.5 in^2 (ca. 29.0 cm^2)	25	150	5
3.8 in^2 (ca. 24.5 cm^2)	30	180	6
3.2 in^2 (ca. 20.6 cm^2)	35	210	7
Total		660	

Six weaning cages were studied at each density.

and perhaps other micro-environmental parameters.

Testosterone assays. The assay procedure has been described elsewhere (14). Variations in urine production were indexed by creatinine measurement. Two-fold dilutions of creatinine standard (Sigma Chemical Co., St. Louis, Mo.) from 100 to 3.12 μg/ml were used, and distilled water was used as 0 μg/ml. All urine samples tested for creatinine were diluted 1:45.

Urinary testosterone concentrations were determined using a capture enzyme-linked immunosorbent assay. The capture antibody, T R156/7 (Department of Population Health and Reproduction at the University of California, Davis), was diluted 1:10,000. Urine samples assayed for testosterone were diluted 1:6 and were measurable in the sensitive range of a standard curve. Two quality control urine samples were prepared by spiking mouse urine with testosterone and diluting them to measure at 30% and 70% binding (the low and high ends of the sensitive range of the standard curve). The testosterone standard (Sigma Chemical Co., St. Louis, Mo.), samples, or controls were added to each well along with horseradish peroxidase-conjugated T R156/7 (Department of Population Health and Reproduction at the University of California, Davis) diluted 1:50,000.

The testosterone standards have a range of 1250 to 2.4 pg/well with a sensitivity of 48 pg/ml of urine. The average interplate co-efficient of variation (CV) for the anti-testosterone R156/7 antibody is 6.8, whereas the intraplate CV is 7.1. The recovery for the testosterone assay is 99.0 ± 5.8%. Cross reactivities for antibody R156/7 are as follows: testosterone, 100%; 5a-dihydrotestosterone, 57.4%; androstenedione, 0.27%; and androsterone, dehydroepiandrosterone, cholesterol, β-estradiol, progesterone, and pregnenolone, < 0.05%.

Within each cage type, density, and sex, urine was collected and pooled from all mice in a given cage. Four cages per type and density were assessed at each interval.

Histologic evaluation of nasal passages and eyes for ammonia toxicity. Mice were euthanized with CO_2 at study termination, and the nasal passages and eyes of selected mice from each housing density in the 4-week study were collected, fixed for 24 h in Bouin's fixative (15 parts saturated aqueous picric acid, 5 parts 40% aqueous formaldehyde, 1 part glacial acetic acid), and washed multiple times in running water. Five levels of nasal passages, sectioned at 5 μm and stained with hematoxylin and eosin, were examined. Eyes were fixed, plastic-embedded, and processed as described (26).

Statistical analysis. A power analysis was conducted prior to study initiation. The numbers of mice and cages (for each cage type) were greater than the minimum required to yield a power of 80%. Analysis of covariance (ANCOVA) was used to test for differences in environmental variables (response) among four housing densities (treatment) while controlling for time effects (covariate). The interaction effect (density*time) was included in the initial analyses to test for different slopes among the density treatments. If not significant, the interaction term was dropped, and the ANCOVA was performed using only the treatment (density) and covariate (time). The data were $\log_e$-transformed to stabilize variances when necessary.

Analysis of covariance was also used to test for differences in mean testosterone levels (response) among the four housing densities (treatment 1) and two genders (treatment 2) while controlling for time (in weeks) effects (covariate). A backwards model selection process was used to identify the best fitting ANCOVA model. Initially, the full factorial ANCOVA model was tested (three main effects, three two-way interactions, and the three-way interaction). Non-significant interaction terms were removed one at a time, and the ANCOVA re-run with the reduced number of effects, until only the main effects and significant interaction effects remained. Tests were performed separately for each cage type. All analyses were performed using JMP (Version 5.0.1.2, The SAS Institute, Inc., Cary, N.C.). All tests were evaluated using $\alpha = 0.05$.

Results

Eight-Week Study: C57BL/6J Mice Housed in Three Cage Types

Animal health. All B6 mice that began the study survived, and we did not observe any aggressive behavior or injured mice. There were no significant differences in rates of weight gain or consumption of food and water among density groups, although males consumed more food and water and, as expected, became heavier than females. The mean (± standard error [SE]) weight of mice at the termination of this experiment was 20.4 ± 0.6 g for females and 29.8 ± 0.8 g for males.

Young female B6 mice develop alopecia more frequently than males (S. L. Mabus and A. L. Smith, unpublished data). Hair loss is frequently the result of dominance or co-dominance behavior and is noticeable by about 8 weeks of age. The incidence of alopecia among B6 female mice used in this study was relatively low (varying from 0% to 6% per treatment group) and was unrelated to cage type or housing density. Ulcerative der-

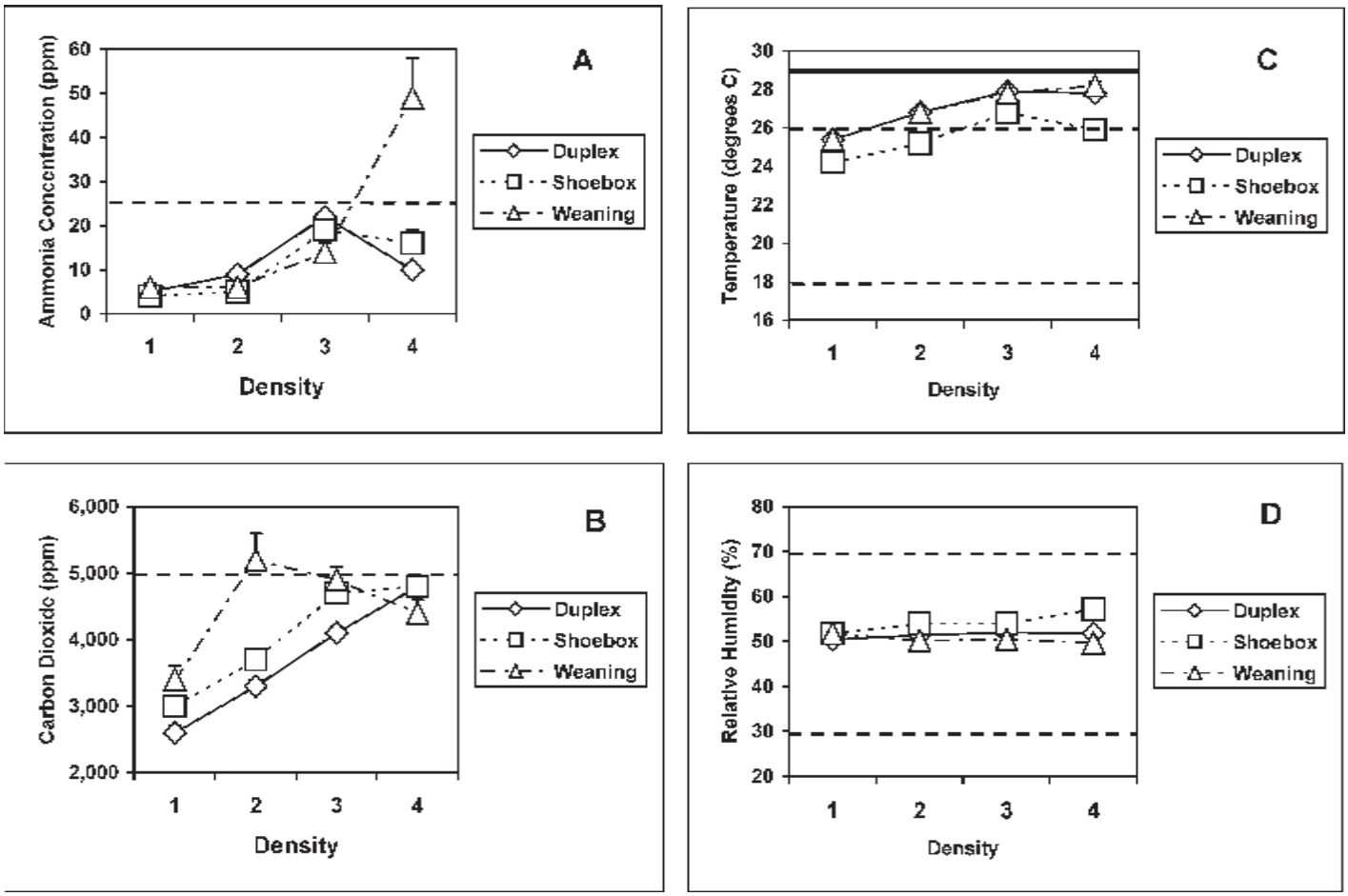

Figure 1. Least squares (i.e., adjusted) mean (± SE) values for various parameters for each cage type and density treatment level for C57BL/6J male mice. (A) NH_3. Horizontal dotted line is the maximum OSHA-allowed human workplace exposure during an 8-h day of a 5-day work week. (B) CO_2. Horizontal dotted line is the maximum OSHA-allowed human workplace exposure during an 8-h day of a 5-day work week. (C) Temperature. Temperature range recommended in the *Guide for the Care and Use of Laboratory Animals* is 18°C to 26°C (dotted horizontal lines). The maximum allowed temperature is 29.4°C (solid horizontal line). (D) Humidity. The humidity range recommended in the *Guide for the Care and Use of Laboratory Animals* is 30% to 70% (dotted horizontal lines).

matitis, observed with some frequency by investigators using older B6 mice, was not seen.

Micro-environment in cages housing C57BL/6J male mice. (i) Ammonia. Ammonia concentrations in duplex, weaning, and shoebox cages were highly variable among density levels and were $\log_e$-transformed for the ANCOVA. Ammonia levels were significantly affected by density (Fig. 1A). Adjusted mean ammonia levels for duplex cages housing densities 1, 2, and 4 were less than or equal to 10.0 ppm, whereas the adjusted mean for density 3 was 21.7 ppm. Adjusted mean values for shoebox cages ranged from 5.3 ppm (density 1) to 18.9 ppm (density 3). Adjusted values for weaning cages ranged from 7.0 ppm (density 2) to 49.0 ppm (density 4). The interaction effects for duplex ($P = 0.49$), shoebox ($P = 0.14$), and weaning ($P = 0.34$) cages were not significant, indicating the regression on the covariate (time) did not have different slopes for different treatment (density) levels.

(ii) Carbon dioxide. In general, carbon dioxide concentrations increased with increasing densities (Fig. 1B). The only exception was a peak of CO_2 levels in weaning cages at density 2, which then declined at higher densities; despite this peak, there were no significant differences in CO_2 concentrations among densities 2, 3, and 4. Mean CO_2 levels varied two-fold ranging from 2,733 to 5,349 ppm. The density–time interaction was not significant for duplex ($P = 0.59$), shoebox ($P = 0.91$), or weaning ($P = 0.44$) cages.

(iii) Temperature. Temperature increased with increasing density, but mean temperatures varied ≤ 4°C (24.3°C to 28.3°C; Fig. 1C) and did not exceed the recommendation in the *Guide*. The density–time interaction effect was not significant for duplex ($P = 0.97$), shoebox ($P = 0.33$), or weaning ($P = 0.66$) cages housing male mice.

(iv) Humidity. Mean RH was relatively constant across densities for each cage type (Fig. 1D). Only shoebox cages showed a statistically significant difference in RH among densities after adjustment for the covariate time (51.7% for density 1 versus 56.9% for density 4). The density–time interaction effect was not significant for duplex ($P = 0.94$), shoebox ($P = 0.44$), or weaning ($P = 0.92$) cages.

Urinary testosterone concentrations for male and female C57BL/6J mice. Neither density nor gender had an effect on urinary testosterone levels of mice housed in any of the three cage types.

(i) Duplex cages. The ANCOVA, with the three main effects (density, gender, week) and the gender–week interaction effects,

was significant (F = 5.1, $P < 0.0001$; $R^2 = 0.21$). However, this significance resulted from differences in the covariate week as evidenced by the fact that urinary testosterone levels (mean ± SE) in female mice significantly increased from 1.82 ± 1.10 (baseline) to 4.35 ± 2.37 ng/mg of creatinine by the end of the 8-week study, and those in male mice decreased (not significantly) from 2.86 ± 1.16 to 1.87 ± 0.91 ng/mg of creatinine by 8 weeks.

(ii) Shoebox cages. The ANCOVA, with the three main effects and the gender–week interaction effect, was significant (F = 8.0, $P < 0.0001$; $R^2 = 0.24$), resulting from differences in the covariate week and the interaction gender–week. Urinary testosterone levels (mean ± SE) of female mice increased from 1.29 ± 0.86 (baseline) to 3.96 ± 1.22 ng/mg of creatinine (8 weeks), whereas those of male mice decreased from 3.19 ± 1.06 (baseline) to 1.54 ± 0.71 ng/mg of creatinine (8 weeks). Both changes were statistically significant ($P < 0.05$).

(iii) Weaning cages. The ANCOVA model with the three main effects was significant (F = 4.9, $P < 0.0003$; $R^2 = 0.14$), but this significance was accounted for completely by the covariate week (F = 20.2, $P < 0.0001$). A single regression described the testosterone data from the weaning cages for all densities and both genders. There were not significant differences in male or female mean urinary testosterone levels between the baseline and week 8 samples. Unlike the results for males in duplex and shoebox cages, urinary testosterone levels increased for males between baseline and 8 weeks, although not significantly. None of the interaction effects were significant for the ANCOVA.

Four-Week Study: C57BL/6J Mice Housed at Higher Densities in Weaning Cages

Because we observed no deleterious effects of housing 20 C57BL/6J mice for at least 8 weeks in weaning cages (5.6 in^2 per mouse), we followed up with a 4-week study that evaluated the same parameters for 20, 25, 30, or 35 mice per weaning cage (down to 3.2 in^2 per mouse). We monitored the micro-environments of both male and female mice twice weekly during this study, and we also assessed both the noses and eyeballs of selected mice microscopically at study termination. Soon after this 4-week study was initiated, three mice were culled due to malocclusion and/or hydrocephaly (fairly common in this inbred mouse strain). One additional mouse was found dead but was not necropsied because of extreme autolysis. Each of these four mice was replaced to maintain the original housing densities.

Animal health. No effect of density on the rate of weight gain was observed and food and water consumption was not different among the densities. Density did affect hair loss (alopecia) in female mice, which developed hair loss in one of six cages at density 4, two of six cages at densities 5 and 6, and five of six cages at density 7. Male B6 mice developed hair loss in one of six cages at each of the four densities.

Micro-environment in cages housing C57BL/6J male or female mice. (i) Ammonia. Ammonia concentrations (mean ± SE) increased significantly ($P < 0.05$) with each increase in housing density (12.6 ± 1.1 ppm, 20.7 ± 1.1 ppm, 43.4 ± 1.1 ppm, and 139.8 ± 1.1 ppm at densities 4, 5, 6, and 7, respectively). Average ammonia values were higher for males compared with females at all but the lowest density (data not shown). The overall ANCOVA model was significant (F = 45.7, $P < 0.0001$; $R^2 = 0.51$).

Nasal passages from selected mice in each density group were examined microscopically at study termination for ammonia damage and were found to be normal. Eyes from 13 randomly chosen male mice were examined. The range of ammonia concentrations to which they had been subjected was 23 ppm to 410 ppm, including 5 mice from cages containing 198 ppm to 399 ppm of ammonia. All of the examined eyes were histologically normal.

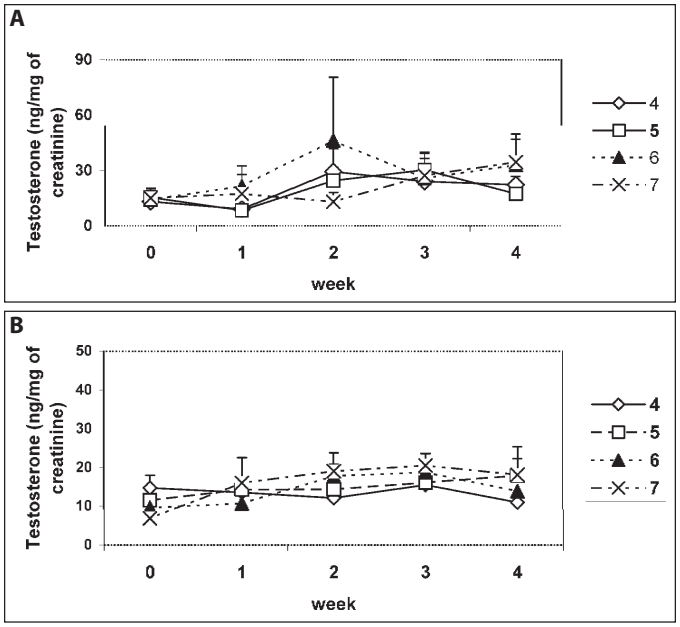

Figure 2. Testosterone concentrations (ng/mg creatinine; mean ± SE) for male (top panel) and female (lower panel) C57BL/6J mice. Data are shown for each density (4, 5, 6, and 7) over time (baseline through 4 weeks)

(ii) Carbon dioxide. CO_2 differences among the densities were not significantly different. Least squares means were greater for males (4,335 ppm) than females (3,103 ppm; $P < 0.05$). The overall ANCOVA model was significant (F = 16.9, $P < 0.0001$; $R^2 = 0.28$).

(iii) Temperature. Least-squares mean temperatures (± SE) were significantly higher for densities 5, 6, and 7 (28.3°C ± 0.2°C, 28.8°C ± 0.2°C, and 28.4°C ± 0.2°C, respectively) compared with density 4 (26.6°C ± 0.2°C). The overall ANCOVA model was significant (F = 44.1, $P < 0.0001$; $R^2 = 0.5$).

(iv) Humidity. The least-squares mean RH value (± SE) for the highest density (density 7, 57.1% ± 1.0%) was significantly higher than those of the two middle densities (density 5, 53.0% ± 1.0%; density 6, 52.9% ± 1.0%) but not different from that of the lowest density (density 4: 56.3% ± 1.0%). The overall ANCOVA model was significant (F = 71.1, $P < 0.0001$; $R^2 = 0.56$).

Urinary testosterone concentrations for male and female C57BL/6J mice. Mean urinary testosterone levels were unrelated to housing density (Fig. 2) and were higher for males than females ($P = 0.002$). The mean baseline concentration for males was 14.4 ± 4.1 ng/mg of creatinine, which increased to 26.8 ± 12.6 ng/mg by the end of the fourth week. For females, the baseline was 10.7 ± 3.9 ng/mg of creatinine and increased to 15.2 ± 5.2 by week 4. The week effect was significant ($P = 0.05$).

Discussion

On the basis of gross measures, the health and well-being of the mice used in these studies were not affected by cage type or housing density. There were no significant differences among mice housed in three cage types, at any of seven densities, in

growth rates or food and water consumption. We did not observe aggressive or injurious behavior, and all mice survived the 8-week period of the first study. Three mice were culled early in the 4-week study due to common B6 mouse anomalies, and one mouse was found dead with no obvious bite wounds. The incidence of alopecia among B6 female mice ranged from 0% to 6% in the 8-week study and was not associated with a particular cage type or housing density. The incidence of alopecia in the 4-week study was density-dependent, with 5 of 6 cages containing affected female mice at the highest density.

In the 8-week study, housing B6 male mice at the highest density (5.6 in^2 per mouse) in duplex and shoebox cages resulted in mean ammonia levels that varied from 5 to 19 ppm. The reason for the spike in ammonia concentration (49 ppm) only in the weaning cages at the highest density is unknown. In-cage CO_2 levels generally increased with density, and there were not apparent differences among the cage types—all reached maximum levels of approximately 5,000 ppm, the maximum allowable U.S. workplace exposure limit during an 8-h (17) or 10-h (16) shift. Increases in CO_2 concentration would be expected at higher densities because CO_2 reflects the amount of respiration occurring within the cage. Mean temperatures generally increased with density and, on average, the difference between the mean high and low temperatures in each cage type was 2.5°C. The *Guide* indicates that temperatures ranging from 18°C to 26°C are recommended for housing laboratory rodents and that a temperature higher than 29.4°C could produce adverse clinical effects. It is not clear whether the recommended temperatures pertain to room temperature or in-cage temperature or whether this recommendation was based on studies using static or ventilated caging systems. In addition, one might expect that adverse effects would be more likely after sudden changes in temperature. In no case did our in-cage temperatures exceed 29°C. In-cage relative humidity was unaffected by cage density except for the difference between density 1 and density 4 in shoebox cages (a difference of 5.2%). The *Guide* indicates that RH can vary widely, from 30% to 70%, and our results were well within that range.

In the 4-week study, average in-cage ammonia concentrations significantly exceeded 25 ppm at densities 6 and 7, reaching 43.4 and 139.8 ppm, respectively. Cages housing male mice had higher concentrations than those housing females. In-cage CO_2 concentrations were independent of density in the 4-week study. Temperatures were higher in cages housing the three highest densities but did not exceed 29°C. Humidity levels were variable, and there was no clear relationship to housing density.

Male urinary testosterone levels either remained relatively constant or declined slightly over 8 weeks in the primary study. For female B6 mice housed in duplex, shoebox, or weaning cages, urinary testosterone levels increased over the course of the 8-week study. Irrespective of cage type, housing density did not influence urinary testosterone output of male or female B6 mice, although week and/or gender did. In the 4-week study, urinary testosterone concentrations from male mice were uniformly higher than those from female mice. As found in the 8-week study, neither housing density nor cage type influenced hormone concentrations. However, hormone levels of male mice increased over the 4-week period, in contrast to the 8-week study in which urinary testosterone levels either decreased or remained constant. Hormone levels from female mice increased in both studies. It should be noted that the testosterone concentrations during the 4-week study were substantially higher than those of the 8-week study. This inter-assay variation is expected and makes it essential that values that will be compared directly must result from simultaneous assays.

Humans exposed to 500 ppm of ammonia can hyperventilate and may complain of nose and throat irritation, and accidental exposure to even higher concentrations of ammonia can result in extensive thermal burns on the lips and conjunctiva, corneal opacities, and edematous, congested lungs with areas of hemorrhage (24). However, even exposure to concentrations as low as 20 ppm may cause discomfort and conjunctival hyperemia (3). Although standards from the Occupational Safety and Health Administration and National Institute of Occupational Safety and Health (15, 18) indicate that workplace exposure to ammonia should not exceed 25 ppm over 8 h or 35 ppm over a 15-min period, two factors can substantially reduce human exposure 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 (23), exposure can be greatly reduced by husbanding rodents on ventilated tables. An additional factor in micro-environmental ammonia concentrations is the type of bedding used to house the animals. We recently surveyed the performance of several types of bedding, and many commercially available bedding materials yielded very low ammonia levels even when mice (B6 males, NOD/LtJ males, and B6 breeder pairs with offspring) were housed for 3 weeks in static cages (25).

There is a sparse literature on the effects of exposure to gaseous ammonia on laboratory rodents, and there is not always good agreement among the studies. However, in view of the literature and our own results, rodents seem able to tolerate higher ammonia concentrations than do humans. Coon and colleagues (5) exposed Sprague-Dawley and Long-Evans male and female rats to several concentrations of ammonia, either repeatedly or continuously. Repeated exposure (8 h per day, 5 days per week, for 6 weeks) to 155 or 770 ppm of ammonia resulted in no discernable toxic effects, although 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, and 25% of the rats exposed to 262 ppm 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." In long-term studies, continuous exposure to > 400 ppm (455 ppm) of ammonia resulted in the deaths 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. Rabbits exposed continuously to > 400 ppm had opacities over 1/4 to 1/2 of their corneas, and this finding was the basis for our microscopic examination of eyes in the 4-week follow-up study. However, those investigators did not examine the nasal passages of any of their exposed animals (5).

The early literature that addressed the noxious effects on rodents of low ammonia concentrations must be considered in the context of the microbial status of laboratory rodents at that time. For instance, Broderson et al. (2) carefully documented the exacerbation of respiratory mycoplasmosis among rats housed in cages with ammonia levels that varied from 25 to 250 ppm, and this study is often quoted as the basis for controlling ammonia levels in animal facilities. Gamble and Clough (7) referred to some laboratory species being unsuitable for studying inhalant

toxicity because of their "natural incidence of abnormal respiratory histology" and suggested that this might reflect the standard of husbandry in animal facilities. The microbiologic quality of contemporary laboratory rodents is considerably higher than that in the 1950s through the early 1980s, when much of this sort of research was performed. Thus, it may well be that the modern mouse, with much less respiratory disease, can tolerate elevated ammonia levels that were harmful to their forbears. This difference may explain why we were unable to demonstrate any harmful effects of ammonia, even at exposure levels considered very high.

Results of our 8-week study indicate that only the least-squares mean ammonia level in density 4 in weaning cages (49 ppm) exceeded the concentration considered unhealthy for humans. In the 4-week study, ammonia levels were very high (43.4 and 139.8 ppm) in cages housing mice with < 4.5 in^2 (ca. 29.0 cm^2) of floor space. For densities 4 to 6, each 15% to 20% reduction in floor space per mouse resulted in an approximate doubling of the in-cage ammonia concentration: the ammonia concentration was tripled when mice had 3.2 in^2 rather than 3.8 in^2 (ca. 24.5 cm^2) of floor space (a 16% reduction). None of the mice in either of the two studies showed evidence of ammonia toxicity, despite exposure to > 200 ppm in some individual cages in the 4-week study. Although the mice in our studies did not demonstrate behavior suggestive of pain, discomfort or distress, a more detailed behavioral study would be required to determine their condition with certainty. However, given the OSHA workplace standards for humans of 25 ppm (18), the use of ventilated changing tables should be encouraged in mouse rooms. Carbon dioxide levels, temperatures, and RH remained within the limits in the *Guide* in both the 8- and 4-week studies.

The incidence of alopecia among female B6 mice housed with 3.2 in^2 of floor space was very high, although not significantly different from density 4 because of the small number of cages (six) included at each density.

The recommendations in the *Guide* were based on best professional judgment at a time when there was very little peer-reviewed literature on the topic. We are attempting to apply scientific methods to learn what the real floor space needs of mice are. Thus, we recommend housing C57BL/6J male and female mice, aged 4 to 12 weeks, in cages that provide not less than 5.6 in^2 of floor space per mouse. This requirement translates to 9 mice per side of duplex cages, 12 mice per shoebox cage, and 20 mice per weaning cage. Some of the male mice used in the 8-week study weighed as much as 29 g at study termination, and the *Guide* would have those mice housed with 15 in^2 per mouse (10).

Our results and those of others (6, 13, 27) have consistently pointed to the same conclusion: Mice that are housed at higher densities tend to be healthier and less aggressive toward their cage mates. For this reason, the current guidelines need to be re-evaluated in the context of what is known about this social species. Animal care should not be dictated by our anthropomorphic perceptions. If animal care staff, investigators and regulatory bodies are educated regarding husbandry practices that benefit the mice, we might improve the well-being of these social animals at no cost or even a cost savings if more mice are housed per unit area. The role of the *Guide* is to ensure that laboratory animals are well treated and housed in a species-appropriate manner. It is hoped that well-designed and controlled studies will contribute to a revision that more closely approximates best practices.

Our study and those in the literature pertaining to mouse housing density have included only a few inbred mouse strains. Universal provision of the floor space needs of mice may be difficult, and inter-strain variation is to be expected. We have recently used the same protocols described here to evaluate the needs of young adult BALB/cJ, NOD/LtJ and FVB/NJ mice. Preliminary analysis indicates that there are, indeed, differences (A. L. Smith, unpublished data). We also caution that the studies reported here dealt only with young adult B6 mice. Further work is required to ascertain the applicability of this work to breeding colonies in which pheromones play a major role in behavior. Whatever practices are chosen, it is very important that consistent housing densities be used for studies destined for inter-experimental analysis. Our experience is that investigators frequently do use the same numbers of mice per cage as a matter convenience and to facilitate statistical analysis. Lastly, the laboratory animal veterinary staff should be consulted before changes in animal stocking densities are implemented.

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References

1. **Bonamin, L. V. and B. E. Malucelli.** 1995. The influence of social isolation and peripheral innervation on Ehrlich tumor growth in mice. Braz. J. Med. Biol. Res. **28:**557-562.
2. **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.
3. **Chao, T. C. and D. S. Lo.** 1996. Ammonia gassing deaths—a report on two cases. Singapore Med. **37:**147-149.
4. **Chapman, J. C., J. J. Christian, M. A. Pawlikowski, N. Yasukawa, and S. D. Michael.** 2000. Female house mice develop a unique ovarian lesion in colonies that are at maximum population density. Proc. Soc. Exp. Biol. Med. **225:**80-90.
5. **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.
6. **Fullwood, S., T. A. Hicks, J. C. Brown, R. L. Norman, and J. J. McGlone.**1998. Floor space needs for laboratory mice: C57BL/6 males in solid-bottom cages with bedding. ILAR J. **39:**29-36.
7. **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.
8. **Grewal, I. S., A. Miller, and E. E. Sercar.** 1997. The influence of mouse housing density on autoimmune reactivity. Autoimmunity **26:**209-214.
9. **Himms-Hagen, J. and C. Villemure.** 1992. Number of mice per cage influences uncoupling protein content of brown adipose tissue. Proc. Soc. Exp. Biol. Med. **200:**502-506.
10. **Institute of Laboratory Animal Resources.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, D.C.
11. **Les, E. P.** 1968. Cage population density and efficiency of feed utilization in inbred mice. Lab. Anim. Care **18:**305-313.

12. **Lin, A. H., C. K. Castle, G. W. Melchior, and K. R. Marotti.** 1995. The effect of population density on the development of experimental atherosclerosis in female mice. Atherosclerosis **115:**85-88.
13. **McGlone, J. J., D. L. Anderson, and R. L. Norman.** 2001. Floor space needs for laboratory mice: BALB/cJ males or females in solid-bottom cages with bedding. Contemp. Top. Lab. Anim. Sci. **40(3):**21-25.
14. **Muir, C., E. Spironello-Vella, N. Pisani, and D. deCatanzaro.** 2001. Enzyme immunoassay of 17β-estradiol, estrone conjugates, and testosterone in urinary and fecal samples from male and female mice. Hormone Metab. Res. **33:**653-658.
15. **National Institute of Occupational Safety and Health.** 1989. Toxicologic review of selected chemicals. Ammonia. [Online]. Available at http://www.cdc.gov/niosh/pe188/7664-41.html. Accessed 2004 June.
16. **National Institute of Occupational Safety and Health.** 1989. Toxicologic review of selected chemicals. Carbon dioxide. [Online]. Available at http://www.cdc.gov/niosh/pel188/124-38.html. Accessed 2004 June.
17. **Occupational Safety and Health Administration.** 1990. Carbon dioxide in workplace atmospheres. [Online]. Available at http://www.osha.gov/dts/sltc/methods/inorganic/id172/id172.html. Accessed June 2004.
18. **Occupational Safety and Health Administration.** 2002. Ammonia in workplace atmospheres—solid sorbent. [Online]. Available at http://www.osha.gov/dts/sltc/methods/inorganic/id188/id188.html. Accessed June 2004.
19. **Ortiz, R., A. Armario, and J. M. Castellanos.** 1984. Post-weaning differential housing and testosterone secretion in male mice. Experientia **40:**1428-1429.
20. **Peng, X., C. M. Lang, C. K. Drozdowicz, and B. M. Ohlsson-Wilhelm.** 1989. Effect of cage population density on plasma corticosterone and peripheral lymphocyte populations of laboratory mice. Lab. Anim. **23:**302-306.
21. **Peters, A. and M. Festing.** 1990. Population density and growth rate in laboratory mice. Lab. Anim. **24:**273-279.
22. **Sayegh, J. F., G. Kobor, A. Lajtha, and C. Vadasz.** 1990. Effects of social isolation and time of day on testosterone levels in plasma of C57BL/6By and BALB/cBy mice. Steroids **55:**79-82.
23. **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:**487-492.
24. **Silverman, L., J. L. Whittenberger, and J. Muller.** 1949. Physiological response of man to ammonia in low concentrations. J. Ind. Hyg. Toxicol. **31:**74-78.
25. **Smith, E., J. D. Stockwell, I. Schweitzer, S. H. Langley, and A. L. Smith.** 2004. Evaluation of cage micro-environment of mice housed on various types of bedding materials. Contemp. Top. Lab. Anim. Sci. **43(4):**10-15.
26. **Smith, R. S., A. Zabaleta, and S. W. M. John.** 2002. General and special histopathology, p. 265-298. *In* R. S. Smith, S. W. M. John, P. M. Nishina, and J. P. Sundberg (ed.), Systematic evaluation of the mouse eye: anatomy, pathology, and biomethods. CRC Press, Boca Raton, Fla.
27. **Van Loo, P. L. P., J. A. Mol, J. M. Koolhaas, B. F. M. Van Zutphen, and V. Baumans.** 2001. Modulation of aggression in male mice: influence of group size and cage size. Physiol. Behav. **72:**675-683.