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Effects of Trio and Pair Breeding of Mice on Environmental Parameters and Nasal Pathology and Their Implications for Cage Change Frequency

Kelsey C Carpenter,[1] Sarah E Thurston,[1] Mark J Hoenerhoff,[2] and Jennifer L Lofgren[1,*]

According to the *Guide*, cage change frequencies must be considered when cage density requirements are exceeded. We monitored ammonia, carbon dioxide, cage wetness, health status, and breeding parameters of trio and pair breeding cages containing CD1 mice in ventilated and static microisolation caging (4 cages per condition) daily for approximately 6 wk. Minimum cage change frequencies for each condition were determined on the basis of performance data. At 3 d after cage change, static trio and pair cages had average ammonia levels of 74 and 38 ppm. Ventilated cages remained below the 25-ppm threshold reported to be potentially deleterious for mice until at least day 7 after cage change. By 7 d after cage change, ammonia levels had risen to an average of 100 ppm and 64 ppm in static trio and pair cages and to 34 ppm and 20 ppm in ventilated trio and pair cages, respectively. Ammonia levels in ventilated cages continued to rise slowly through day 14 after cage change. CO_2 levels exceeded 5000 ppm in all groups at 2 d after cage change. Pair breeders in ventilated cages took the longest—10 to 14 d—to reach cage wetness threshold scores. On day 7, pups in trio static cages were noted to have decreased and squinted eyes, whereas in ventilated cages containing trios and pairs, these clinical signs were rare to absent. Histologically, there was an increasing incidence and severity of nasal lesions in weanlings with increasing housing density and decreasing ventilation, consistent with nasal epithelial toxicity. Given these parameters, we concluded that under the current husbandry conditions, it may be necessary to change breeders in static cages more frequently than every 7 d. Additional studies are necessary to evaluate the effects of more frequent cage changes on reproductive parameters, given that cage changing is stressful for mice and affects breeding results.

Abbreviations: SP, pair breeders in static caging; ST, trio breeders in static caging; VP, pair breeders in ventilated caging; VT, trio breeders in ventilated caging

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Facilities historically breed mice continuously, with one or 2 dams, their litters, and the sire housed in the same cage. Continuous breeding, or keeping the sire with the dams, takes advantage of postpartum estrus to decrease the time between litters. In this way, facilities can cost-effectively breed mice to meet research needs. However, concerns arise over compromised air quality and adverse health effects with high housing densities. As a result, the 8th edition of the *Guide for the Care and Use of Laboratory Animals* introduced new space recommendations for breeding mice.[35] The recommended minimum floor area of 51 in.^{2} for a dam and her litter requires 117 in.^{2} of floor space for continuous trio breeding with the addition of an adult male and another dam with her litter.[35] Consequently, many standard mouse cages, with floor areas of 60 to 100 in.^{2}, are now too small for trio breeding configurations. As a result, this breeding scheme has become the exception rather than the norm. When *Guide* exceptions are requested, IACUC have had to reevaluate cage change frequencies to maintain acceptable microenvironmental standards and ensure appropriate animal welfare.

Although the *Guide* provides specific space recommendations for housing, decisions regarding the interval between cage changes are left to professional judgment, with collaboration between investigators and animal care staff.[35] Determining cage change intervals involves balancing multiple factors: maintaining acceptable standards in the microenvironment, minimizing stress, and ensuring the quality of both animal and human health.[54] Unfortunately, because of all these factors, the ideal cage change frequency for a given situation can be challenging to determine. More frequent cage changes maintain low pollutant levels and clean bedding. However, frequent cage changes are resource-intensive, stressful for mice, use additional room technician time, and involve allergen exposure.[6,27,53,70] Conversely, less frequent cage changes equate to reduced animal stress by minimally disturbing mice and less frequent handling.[52] Mice pheromones, olfactory cues, and nesting materials have fewer disturbances.[1] Therefore, mice will likely have more successful breeding and are at a lower risk of preweaning pup loss.[25,49,54] However, as the interval between cage changes increases, ammonia levels rise, the bedding becomes increasingly soiled, and suboptimal microenvironmental conditions result.[6,18,54,59,71]

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[1]Refinement and Enrichment Advancements Laboratory (REAL) and [2]In Vivo Animal Core, Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, Michigan
[*]Corresponding author. Email: jennifer.lofgrendvm@gmail.com

The *Guide* describes some microenvironmental standards, whereas others are left to professional judgment. Acceptable room temperatures range from 20 to 26 °C, and relative humidity ranges from 30% to 70%. The *Guide* recommends adequate ventilation to maintain appropriate air quality and dilute gaseous contaminants, but maximums for ammonia and carbon dioxide concentrations are not specified. Instead, a performance standard is described, setting maximal concentrations of waste gases "below levels irritating to mucous membranes."[35] Resulting studies have found inconsistent reports of rodent ammonia tolerance and histopathology at different concentrations.[40,54,63,64] No respiratory pathology was found in mice exposed to 25 or 100 ppm ammonia for 10 d,[54] but other studies have shown histologic lesions in the nasal cavities of mice[13,71] and rats[4] associated with environmental ammonia levels ranging from 25 to more than 200 ppm for 18 to 42 d. Given the cited studies and the recommendations for human exposure to ammonia, many facilities choose ventilation rates and cage change frequencies that maintain ammonia concentrations below 25 to 50 ppm. For carbon dioxide exposure in rodents, many studies use the limit established for humans, which is 5000 ppm averaged over an 8-h workday.[46] Overall, studies to determine acceptable microenvironment standards specifically for mice are sparse.[68]

To be in compliance with federal guidelines, our institution transitioned to primarily pair breeding of mice, with one male and one female. Researchers who wish to continue trio breeding must apply, with a scientific justification, for an exception from the IACUC by documenting the difference in breeding success. Exceptions allowing for trio breeding generally occur with genetically modified mice that are challenging to breed, because trio breeding has a better outcome in terms of more pups weaned compared with pair breeding scenarios.[23,36,73] Housing breeders in static caging is another option, given that IVC use has also been associated with reduced fecundity.[69]

Because trio breeding exceptions can potentially create high-density housing scenarios, we conducted a study to assess air quality and reproductive success in cages with trio and pair breeders in ventilated and static cages to determine optimal cage change frequencies for each scenario. We hypothesized that ventilated cages with paired breeders and litters would maintain ammonia levels below 25 ppm for significantly longer periods of time compared with breeders in static or trio conditions. We also hypothesized that the nasal cavities of weanling mice in static cages with trio breeding would show histologic lesions consistent with prolonged exposure to high ammonia levels.

Materials and Methods

Ethical statement. This study followed the guidelines of the IACUC of the University of Michigan, which approved all of the animal procedures and animal care methods presented here. The IACUC is in full compliance with the 8th edition of the *Guide for the Care and Use of Laboratory Animals*.[35]

Animals. Male and female CD1 mice (age, 12 to 14 wk; Charles River Laboratories, Portage, MI) were housed in pairs (1 male, 1 female) or trios (1 male, 2 females) in autoclaved polysulfone microisolation cages (7 3/4 in. × 12 in. × 6 1/2 in. [19.7 cm × 30.5 cm × 16.5 cm], Allentown Caging, Allentown, NJ). To ensure a high number of pups for optimization of environmental conditions and reliable endpoints, CD1 mice were chosen because this outbred stock has good fecundity and large litter sizes. Cages were either on individually ventilated racks (model MS7115U54MGPSHR, Allentown) or on static free-standing wire shelving racks, with 76 cm × 152.4 cm shelves spaced 38 cm apart vertically (model 3060NS, InterMetro Industries, Wilkes–Barre, PA). Mice were randomly assigned to their housing and breeding conditions and then maintained in that assigned breeding group but crossed between static and ventilated caging in an alternating fashion for a total of 3 rounds of breeding. This crossover design allowed for a reduction in animal numbers and provided 12 data samplings for each of the 4 cage and breeding paradigms.

The IVC used recirculated room air and had 2 separate HEPA filters to provide filtered air to the system while the exhaust module filtered air from the unit. The rack was tested to calculate average air changes per hour after housing mice in this study. Rack ventilation was measured by using cage monitor units (Enviro-Gard, Lab Products) at 40 random locations on the rack. Ventilation rate was 42.0 ± 3.0 air changes hourly across all used rack locations. All cages were on a 12:12-h light:dark cycle (lights on, 0600) in a temperature- and humidity-controlled room. Room temperature was maintained at 72 ± 2 °F (22.2 ± 1.1 °C), and room humidity was maintained at 30% to 70%. Mice had unlimited access to a commercial rodent diet (PicoLab Laboratory Rodent Diet 5L0D, PMI Nutrition International, Brentwood, MO) and reverse-osmosis-purified water through an automatic watering system or as autoclaved water in water bottles (for groups housed in static cages). Mice were housed on approximately 300 mL of a 50:50 blend of 1/4-in. and 1/8-in. irradiated corncob bedding (Anderson's Bed-O'Cobs, Frontier Distributing, Maumee, OH), with 6 g of brown crinkle paper encased in white tea-bag material (EnviroPak, Shepherd Specialty Papers, Watertown, TN).

Colony health was evaluated quarterly by sentinel exposure to dirty bedding. All sentinels were seronegative for mouse hepatitis virus, mouse parvovirus, minute virus of mice, epizootic diarrhea of infant mice, ectromelia virus, Sendai virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, reovirus type 3, lymphocytic choriomeningitis virus, mouse adenovirus, polyoma virus, *Mycoplasma pulmonis*, and cilia-associated respiratory bacillus. Environmental and colony animal PCR testing for fur mites and pinworms were negative also. Mice were allowed to acclimate for 3 wk in same-sex housing prior to breeding.

Data collection. Data were collected daily (between 0700 and 1000), starting when pups were born until pup weaning at 20 to 22 d of age. The room and all cages were monitored daily for humidity and temperature (Traceable Dew-Point/Wet-Bulb/Humidity Thermometer, Fisher Scientific, Waltham, MA). Cages were measured daily for ammonia and carbon dioxide concentrations by using a photoionization detector (MSA Gas Monitors, MSA Altair 5X, Grainger, Lake Forest, IL) that was calibrated to an isobutylene standard (Calibration Gas Mixture, NorLab, Boise, ID) according to the manufacturer's recommendation. The detector recorded air concentration of specific gases in parts per million every 3 to 5 s. A single sampling hose connected to the detector was used throughout the study. The hose was external to the cage and inserted through the backport where the water automatic watering system enters. The tube was purged between samples, and any debris was removed. The ammonia range of detection was 0 to 100 ppm; values greater than or equal to 100 ppm were recorded as 100 ppm. A temperature and humidity data logger probe (EL-USB-2, Easy Log, Lascar Electronics, Erie, PA) was placed inside cages, above the rodent chow in the feeder; measurements were automatically taken every 12 h. The amount of cage bottom with urine-soaked bedding was scored by examining cages from below, according to an institutional standard operating procedure. A cage with a score of 0 had no wet areas, a score of 1 indicated that less

than 25% was wet, and a score of 2 showed that greater than 25% was wet. Cageside health assessments were performed on a daily basis by a veterinarian (KC) or trained technician (ST). Hair coat, eyes, and posture were evaluated. Nine pups were reared per cage, according to the institutional standard operating procedure which allowed for no more than 9 pups past 9 d of age in a trio breeding cage. Additional pups were euthanized by decapitation before 1 d of age. Breeding data collected included the date of birth and number of pups born and number of pups weaned with their sex and weights at the date of weaning. Nests were scored from 0 to 5 by using a previously described 'naturalistic nest score' system.[34] The score indicated the quality of the nest: higher scores indicated higher-quality, more dome-shaped nests, whereas those with lower scores were flatter or dispersed.

Ventilated cages were changed every 14 d and static cages were changed every 7 d. Cages were not opened between cage changes unless food or water was depleted, mice demonstrated health concerns, or early cage change criteria were met. Early cage change criteria included ammonia levels in excess of 100 ppm and bedding that was more than 25% wet.

Histopathology. At 21 to 23 d of age, weanling mice were euthanized in their home cage by exposure to carbon dioxide at a fill rate of approximately 10% to 30% (1.0 to 3.0 L/min). The removal of a vital organ was the secondary method of euthanasia. After euthanasia, lungs from weanling mice were insufflated with 10% buffered formalin and then immersed in fixative. After removal of the mandible and overlying soft tissues, skulls were fixed in 10% buffered formalin for at least 72 h prior to decalcification (Cal-EX, Fisher Scientific, Pittsburgh, PA). Once decalcified, 3 transverse sections were made by using anatomic landmarks according to guidelines published by the European Registry of Industrial Toxicology Animal (RITA) working group for young mice, as follows.[60] One section was taken immediately posterior to the front incisors (level I), a second section was taken at the level of the incisive papilla (level II), and a third section through the middle of the second molar teeth (level III). Paraffin-embedded sections (5 µm) were mounted on glass slides and routinely stained with hematoxylin and eosin for microscopic evaluation. Sections of lungs and nasal cavity from weanlings ($n = 6$ per housing and breeding group; 3 males and 3 females) were examined by a board-certified veterinary pathologist (MJH). In accordance with best practices for toxicologic histopathology,[9] the pathologist was nonblinded. The scoring method for each section was based on distribution of each lesion within the nasal cavity, as follows: 0, lesions above background levels not observed; 1 (minimal), focal to multifocal lesions involving less than 5% of the epithelium or tissue; 2 (mild), multifocal lesions involving 5% to 25% of the epithelium or tissue; 3 (moderate), multifocal to locally extensive lesions involving 26% to 50% of the epithelium or tissue; and 4 (severe), locally extensive to diffuse lesions involving more than 50% of the epithelium or tissue. Adult mouse histopathology was not assessed as a result of the crossover study design, where adults were exposed to both static and ventilated housing.

Statistical analysis. Average ammonia levels and nasal pathology scores were calculated by group, and standard errors are depicted in each corresponding figure. Ammonia, carbon dioxide, temperature, and humidity levels were compared between groups by using 2-way repeated-measures ANOVA. In cases where ANOVA showed a difference between groups, posthoc analysis (Tukey Highly Significant Difference) was used to determine groups that differed. In all cases, P values less than 0.05 were considered indicative of a statistically significant difference. For statistical analysis of histopathology, groups were compared by using a Kruskal–Wallis test for nonparametric data, with Bonferroni correction. Analyses were conducted by using Prism version 7.04 (GraphPad Software, San Diego, CA).

Results

Intracage ammonia and carbon dioxide levels over time. Intracage ammonia and carbon dioxide levels were compared daily after cage change between housing strategies and breeding paradigms (Figure 1 A and B). Because cage biomass is a factor in ammonia levels, results shown are controlled for 9 pups (age, 14 to 22 d). This age range was chosen to evaluate the maximal housing capacity and biomass density with pups nearing weaning age. In general, mean ammonia and carbon dioxide levels were nonsignificantly higher for static cages. Because there were no significant differences between trios and pairs in either static or ventilated housing, differences noted are primarily a result of housing type. Mean intracage ammonia levels increased over time ($P < 0.05$) after cage change for all breeding and housing scenarios. The 25-ppm ammonia threshold was surpassed at 1, 2, and 3 d after cage change for the static trio, static pair, and ventilated trio mice, respectively. Unlike ammonia, carbon dioxide did not increase over time for the different groups. All groups had carbon dioxide levels greater than the 5000 ppm threshold as soon as 1 d after cage change. In ventilated cages, ammonia and carbon dioxide levels did not significantly change from days 6 to 14 after cage change (data not shown).

Effects of housing status and breeding paradigm on temperature, relative humidity, cage wetness, and nesting complexity. Mean intracage temperatures were compared daily after cage change (Figure 1 C). Mean temperatures across all time points for each scenario were as follows: 24.9 °C in static trio cages, 24.5 °C in static pair cages, 24.1 °C in ventilated trio cages, and 23.3 °C in ventilated pair cages. The mean intracage temperatures for all cages did not exceed the recommended macroenvironmental temperature (26.1 °C). Mean intracage relative humidities were compared daily after cage change (Figure 1 D). In general, static cages were significantly ($P < 0.05$) more humid than ventilated cages and exceeded the maximum of 70% relative humidity. Intracage temperature and relative humidity remained stable over time for the 4 scenarios (±3 °C and 5%, respectively). For ventilated cages, intracage temperature and relative humidity did not significantly change from days 6 to 14 after cage change (data not shown). Cage wetness (no. of days until more than 25% of the corncob bedding was wet) was compared between housing strategies and breeding paradigms (Figure 1 E). The bedding took significantly ($P < 0.05$) less time to become more than a 25% wet in static cages than in ventilated housing. On average, static cages took 6 to 7 d to become 25% wet whereas ventilated trio cages took only a few days longer. Ventilated pair cages took 11 to 14 d or never reached the wetness threshold before the 14-d cage change. Nest complexity was not affected by breeding paradigm or cage ventilation (data not shown).

Reproductive success and clinical signs according to housing and breeding paradigm. Pup weights at weaning were compared between housing strategies and breeding paradigms (Figure 1 F). Normalized weights (weight in grams/age of pup in days) for each pup were calculated, and those pups born to paired breeders in static cages were significantly ($P < 0.05$) smaller than pups in the other 3 housing and breeding paradigms. Pups born to trio breeders in ventilated cages were significantly ($P < 0.05$) larger than pups born to pair breeders in ventilated cages. However, average weaning weights in all housing and breeding paradigms were within published reference ranges

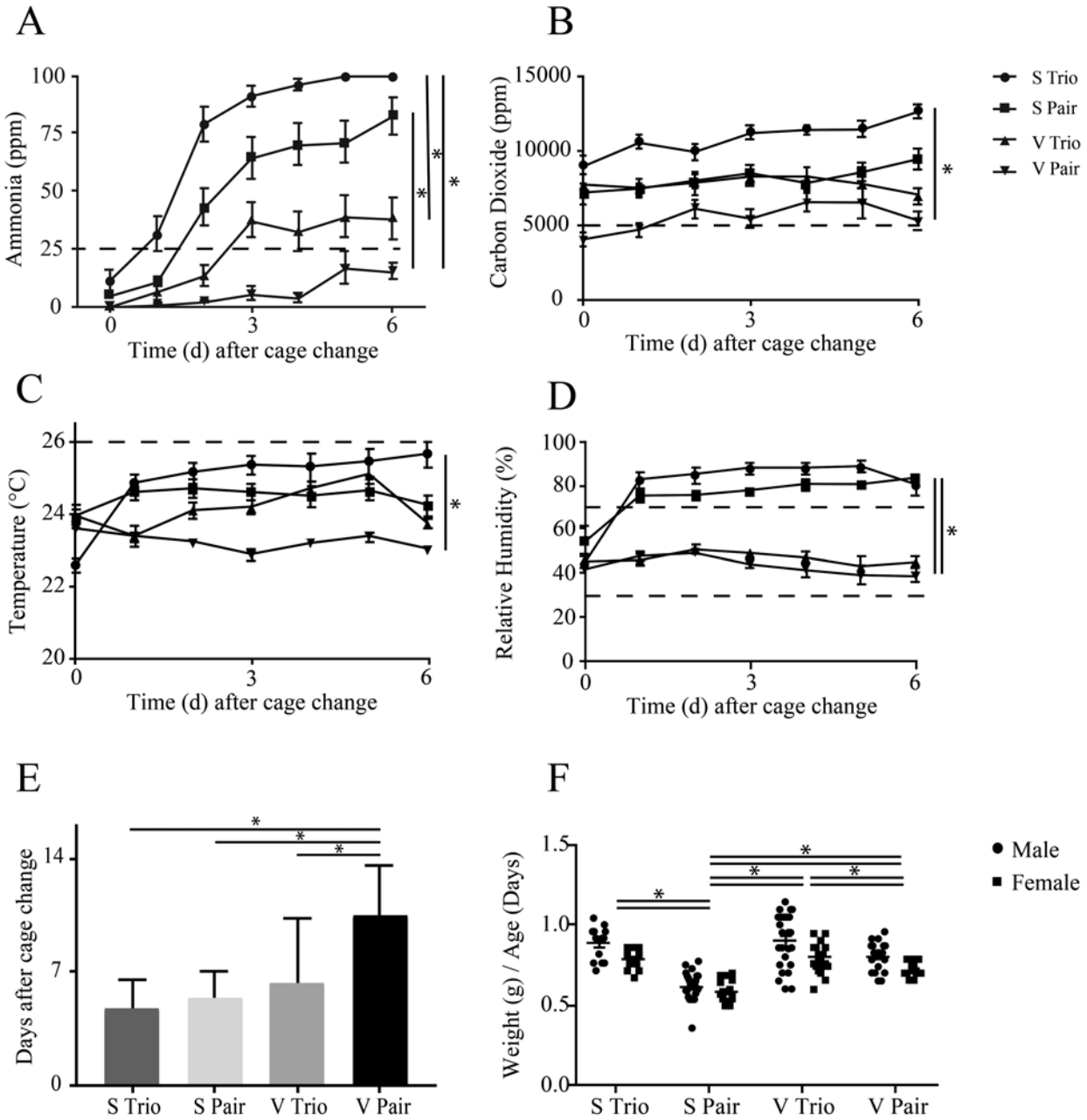

Figure 1. Microenvironments and weanling weights. Microenvironment parameters across all housing and breeding conditions between days 0 and 6 after cage change are shown. While measurements were taken daily throughout the entire study, comparisons between static and ventilated cages are focused on days 0 to 6 as static cages are changed every 7 d. In addition, with the exception of data shown in panel E, no significant changes in these parameters were identified between days 7 and 14 in ventilated cages. Graphs depict (A) intracage ammonia levels, (B) intracage carbon dioxide levels, (C) intracage temperature, (D) intracage relative humidity, (E) number of days after cage change until more than 25% of the corncob bedding was wet, and (F) normalized pup weights at the time of weaning (days 21 to 23). Graphs A through E report data as mean ± SEM. S Trio, trio breeders housed in static caging; S Pair, pair breeders housed in static caging; V Trio, trio breeders housed in ventilated caging; V Pair, pair breeders housed in ventilated caging. Bars indicate significant (*, $P < 0.05$ [2-way ANOVA]) differences between groups over time. Double bars in panel D show significant differences between multiple groups (static groups compared with ventilated groups). Dashed lines show threshold values.

for age-matched CD1 mice.[7] No significant difference was observed in litter size, litters per dam, and pups born per dam (data not shown). In contrast to other groups, trio breeders in static caging showed abnormal behaviors (blepharospasm and decreased movement of pups) 6 to 7 d after cage change when pups were 14 d or older in age.

Effects of increased animal density and static housing on nasal lesions at the time of weaning. Histologically, nasal lesions in weanlings increased in severity and incidence with exposure to increasing concentrations of ammonia (Figure 2). Whereas breeding density (pair compared with trio) did not appear to significantly affect nasal lesion severity, mean histology scores were increased significantly ($P < 0.05$) for mice in static cages compared with ventilated cages for nasal lesions associated with injury, including suppurative inflammation, olfactory and respiratory epithelial atrophy, degeneration or necrosis, respiratory epithelial hyperplasia, respiratory epithelial squamous metaplasia, and turbinate lysis (Table 1 and Figure 2). Minimal

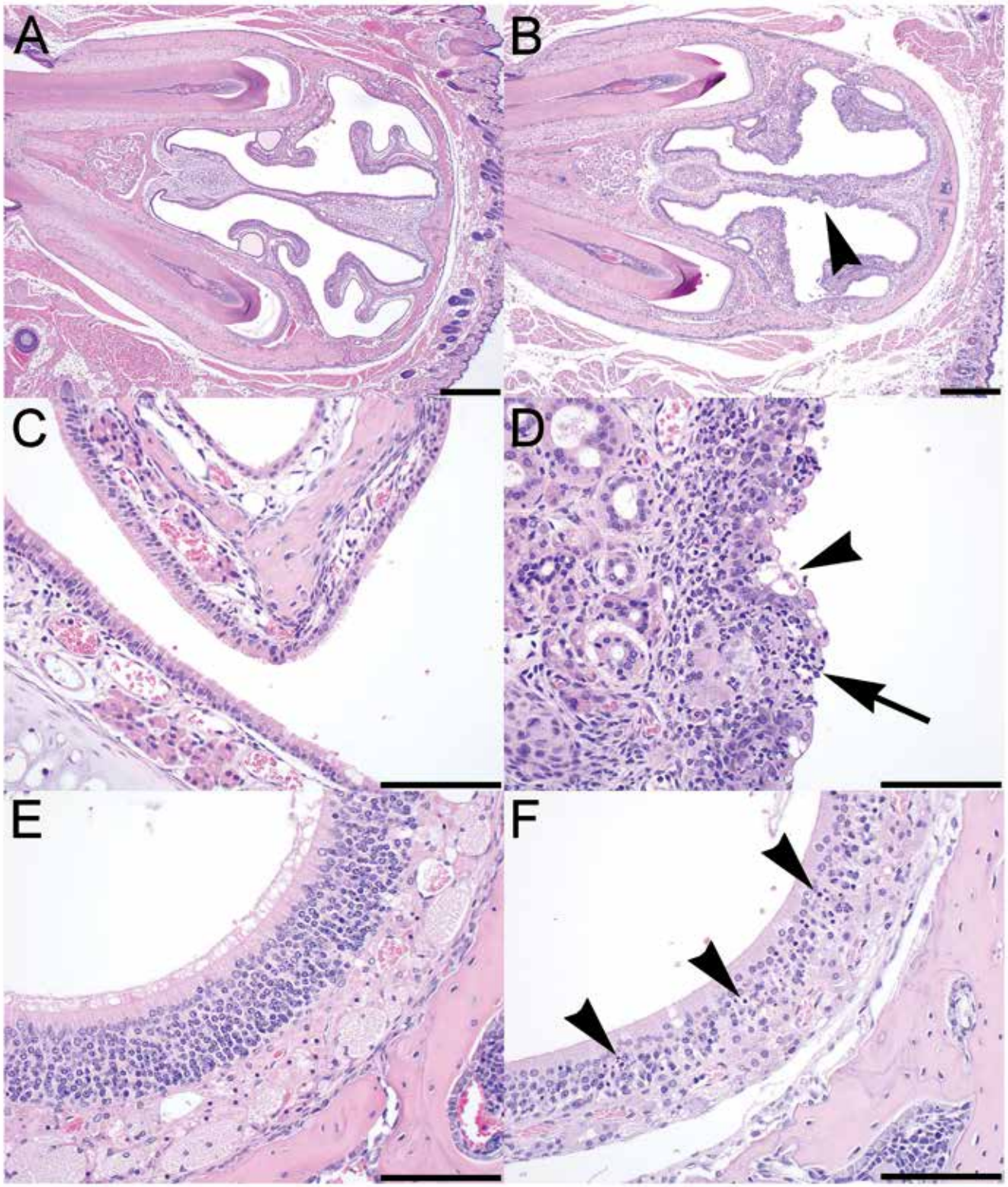

Figure 2. Nasal histopathology. Representative examples of nasal histopathology from weanling mice in (A, C, and E) ventilated cages with pair breeders and (B, D, and F) static cages with trio breeders. In affected animals, (B) the surface of the nasal epithelium was irregular and pitted (arrowhead), due to (D) marked respiratory epithelial degeneration and necrosis (arrowhead) and marked suppurative to pyogranulomatous inflammatory infiltrates (arrow). In sections of olfactory epithelium, (F) affected animals showed moderate to marked olfactory epithelial atrophy and necrosis evidenced by thinning of the olfactory epithelium with scattered shrunken cells with pyknotic nuclei (arrowheads). Magnification, 4× (A and B), 40× (C through F).

and focal background lesions were observed in ventilated pair weanlings and were graded as baseline (mean histology score of 0), with which comparisons with other groups were made. Weanlings from static trio groups had the most severe lesions overall, followed by weanlings from static pairs, whereas weanlings from ventilated trio groups had the least severe lesions,

Table 1. Mean severity scores for histopathologic lesions in weanlings from ventilated and static breeding cages

	Ventilated caging		Static caging	
Histopathologic lesion	Pairs	Trios	Pairs	Trios
Inflammation, suppurative	0	0.2	1.3	3.0
Olfactory epithelium, atrophy	0	0.2	2.5	2.3
Olfactory epithelium, necrosis	0	0.0	2.5	1.7
Respiratory epithelium, hyperplasia	0	0.5	1.2	2.7
Respiratory, metaplasia, squamous	0	0.0	1.0	1.5
Respiratory epithelium, atrophy	0	1.3	1. 7	3.0
Respiratory epithelium, necrosis	0	0.0	1.8	1.8
Turbinate, bony lysis	0	0.0	0.3	1.7
Goblet cell hyperplasia	0	0.2	0.2	0.7

n = 6 weanlings evaluated per group

The scoring method for each section was based on the distribution of each lesion within the nasal cavity: 0, no lesions in excess of background levels; 1 (minimal), focal to multifocal lesions involving <5% of the epithelium or tissue; 2 (mild), multifocal lesions involving 5% to 25% of the epithelium or tissue; 3 (moderate), multifocal to locally extensive lesions involving 26% to 50% of the epithelium or tissue; and 4 (severe), locally extensive to diffuse lesions involving >50% of the epithelium or tissue.

compared with weanlings from ventilated pairs. Lesions were most severe in the most rostral section of the nasal cavity and decreased in severity and distribution caudally toward the nasopharynx, consistent with direct toxicity of an inhalant to the nasal cavity. Lesions tended to occur at specific anatomic locations, including the respiratory epithelium lining the nasal septum, the transitional epithelium at the nasoturbinates, maxilloturbinates, and lateral wall of the nasal cavity, and the olfactory epithelium lining the dorsal meatus of the nasal cavity. There were no lesions observed in the lungs, consistent with other research.[16]

Suppurative inflammation (rhinitis) was characterized by infiltration of the submucosa by variable numbers of leukocytes, predominantly neutrophils (Figure 2). Respiratory and olfactory epithelial atrophy was observed as an overall decrease in cell height and volume, whereas respiratory epithelial hyperplasia was characterized by increased numbers of respiratory epithelial cells, cellular crowding and piling, and undulation and folding of the epithelial surface. Respiratory and olfactory epithelial degeneration and necrosis was characterized by cytoplasmic vacuolation, condensation and shrinkage, nuclear pyknosis and karyorrhexsis, and epithelial erosion, ulceration, and loss (Figure 2). Olfactory epithelial degeneration and necrosis was observed predominantly in the dorsal meatus of the nasal cavity at level II. Finally, squamous epithelial metaplasia of respiratory epithelium was observed as a transition of normal respiratory epithelium to a flattened, squamous phenotype.

Discussion

The ideal cage change frequency must balance animal welfare with microenvironmental conditions and resource availability. In breeding situations, optimizing reproduction and pup wellbeing are additional important considerations. To help determine cage change frequency for various housing and breeding scenarios, we measured several parameters. Our performance-based criteria were measurements of intracage ammonia and carbon dioxide concentrations, temperature and humidity, pup weanling weight and general health parameters, and histopathology of the nasal cavity of weanlings in each breeding and housing paradigm. Our goal was to determine whether trio breeding scenarios would necessitate more frequent cage changes in static or ventilated cages compared with pair breeding scenarios.

Ammonia, a primary waste product in a closed housing environment, is a severe irritant to the respiratory tract and ocular mucous membranes.[47] Ammonia levels increase over time in rodent cages due to accumulating urine and feces. As ammonia levels rise, cages generally become wetter in cages with corncob bedding.[72] Microorganisms in feces or bedding convert urea to ammonia due to the enzyme urease.[58] Currently, ammonia exposure standards have not been established for rodents, so human standards are frequently used. The National Institute for Occupational Safety and Health set an 8-h time-weighted average exposure limit of 25 ppm, with a maximum exposure of 50 ppm.[45] The Occupational Safety and Health Administration has a permissible exposure limit of 50 ppm for ammonia, averaged over an 8-h workday.[46] Therefore, 25 ppm is generally used as a guideline for the maximum threshold for exposure in rodent cages.[14,28,51] However, some disagreement exists over whether the threshold should be higher or lower. Some argue for a lower ammonia threshold given that, unlike humans, rodents are constantly exposed to ammonia[39,48] and are obligate nasal breathers.[24] Conversely, proponents of higher ammonia thresholds emphasize that wild rodents live in crowded underground burrows with limited airflow.[28,59] Other authors report that exposure of laboratory mice to ammonia levels far greater than 25 ppm does not appear to be harmful, given the lack of observed indications of ammonia toxicity.[63,64] Furthermore, mice did not find exposure to 110 ppm ammonia aversive during a preference testing experiment.[28]

In addition, conflicting results exist with regard to the effects of increased ammonia on the rodent respiratory tract.[13] Some studies suggest high ammonia concentrations may contribute to negative health effects in mice, including lesions in nasal and olfactory mucosa and altered biologic responses.[5,26,41,43,71] In 2 studies, the nasal epithelium was affected adversely by exposure to inhaled ammonia and lesion development depended on both ammonia concentration and the duration of exposure.[43,71] Clear nasal cavity pathology was seen after 7 d in static cages where ammonia levels ranged from 50 to 264 ppm at 1 d after cage change.[18]

In the current study, weanling mice in static cages exposed to ammonia concentrations exceeding 25 ppm for at least 15 consecutive days prior to weaning developed inflammatory and degenerative lesions in the nasal cavity. These results were consistent with other studies in rodents exposed to inhaled ammonia or pollutants from soiled bedding.[2,5,13,20,30,31,44,50] The

severity of lesions was most severe in the rostral nasal cavity (often involving lysis of turbinate bone in most severe cases), consistent with an inhaled irritant such as ammonia.[2,10,12,15,30] Ammonia levels in static cages housing trios or pairs with their offspring were on average higher than 25 ppm on day 2 after cage change. Although additional studies are warranted to establish a specific upper limit for inhaled ammonia in mice, the results of our current study show that increasing housing density and decreased airflow (static caging) increased the incidence and severity of lesions within the nasal cavity of weanling mice.

Despite the significant nasal lesions, clinical signs were apparent in only a few animals. This finding is similar to other reports, in which observers noted few overt clinical signs in mice with nasal lesions due to ammonia exposure.[63,64] Lesions in mice with rhinitis are most often clinically silent, and clinical signs, such as squinting and rubbing of the eyes, might be missed due to subtlety of response or observations made during the light cycle when mice are intentionally aroused from sleep.[71] Typically, mice with severe rhinitis do not develop significant weight loss and show no obvious signs of discomfort or illness.[71] Rodents exposed to higher ammonia concentrations are, however, at a greater risk of developing *Mycoplasma pulmonis* infections.[61]

Other than ammonia, carbon dioxide is the other primary gaseous waste product in closed housing environments and similarly lacks official animal limits for exposure. The present human occupational exposure limit is 5000 ppm for an 8-h time-weighted average,[29] but no limits exist for rodents. Within mouse cages, levels range from 1000 to 6000 ppm, depending on ventilation, lid type, and sampling location.[37,47,55,62,63] In humans, headache is reported at concentrations ranging from 2500 to 5000 ppm and loss of consciousness at 100,000 ppm.[29] Extrapolation to mice is complicated by species differences; wild rodents, for example, inhabit burrows with limited ventilation that may have levels as high as 14,000 ppm.[13,67] Therefore, some researchers suggest a much higher acceptable experimental limit of 15,000 ppm.[37] At higher levels, carbon dioxide is an approved method of euthanasia for rodents[38] and can lead to pulmonary hemorrhage and lesions in the upper respiratory tract.[11,19,57] Nasal cavity lesions have been limited to nasal hemorrhage with no damage to nasal mucosa.[19]

The carbon dioxide concentrations recorded in our current investigation were higher than those published in other studies.[7,43,48,56,62] These increased values may reflect the location of measurements, the overall cage design and lid tightness, or the high cage densities in this study. Previous work has demonstrated carbon dioxide levels of less than 3000 ppm between 1 and 9 d after cage change.[64] Other studies have reported carbon dioxide levels below 2500 ppm with ventilation rates of 30 air changes hourly.[54] In another study, levels ranged from approximately 945 ± 2200 ppm for pair-mated mice and from 1400 ± 3660 ppm in trio-mated mice.[55] Carbon dioxide levels are directly related to the number of animals in the cage and increased biomass. Significantly higher carbon dioxide concentrations were seen in cages with litters compared with those without litters.[13] Therefore, it is not surprising that cages with trio breeders and static ventilation displayed higher carbon dioxide levels throughout the course of the study. Despite prolonged exposure and euthanasia by carbon dioxide, we obtained no findings of pulmonary or nasal hemorrhage.

Intracage temperatures measured in our current study remained within the recommended macroenvironmental range for mice (20 to 26 °C) when mice were housed within ventilated cages.[35] Static cages with trio-mated mice exceeded the 26 °C threshold when pups were older than 14 d as soon as 1 d after cage change. However, debates exist over the optimal housing temperature for mice and are beyond the scope of the current study.[66] The higher temperatures with trio breeding scenarios and older pups are unsurprising, given the larger body mass within the cage and additional heat generation. The data logger in our study was placed in the food hopper above the mice. Given that warm air rises, a more accurate intracage temperature experienced by the mice might have been obtained at the level of the animals. Relative humidity was significantly increased in static cages compared with ventilated cages as early as 1 d after cage change. Other studies have had similar findings with increased ventilated maintaining a drier, less humid cage environment.[55] The *Guide* recommends a room relative humidity of 30% to 70%.[35] Although no guidelines are set for cage level, the *Guide* acknowledges the restricted ventilation in static cages may necessitate adjusted husbandry practices.[35] The dryness of bedding and relative humidity inside the cage is important to animal health because too much moisture in animal cages enhances the proliferation of urease-positive bacteria and increases ammonia production.[8,21,32,42,54,62] Therefore, with static breeding cages, more frequent cage changes or a different type of bedding should be a consideration to decrease intracage relative humidity.

In addition to environmental parameters and clinical signs, we evaluated weanling weight. We found pups born to paired breeders in static cages were smaller, whereas pups born to trio breeders were larger. Other studies have shown laboratory mice raised in communal nests produce heavier pups at weaning.[3,33,65] These findings could be a side effect of alloparenting, where the additional female mouse shares maternal duties such as grooming, nest building, and feeding.[22] Regardless, the increased weanling weights were modest, and the lower weights were still within normal limits for CD1 mice. Other studies have shown similar weanling weights between pups born to trio or paired breeders.[36]

During daily health assessments, neonates and weanlings in static cages with trio breeding sporadically exhibited blepharospasm. Whereas clinical signs such as blepharospasm and conjunctivitis were observed and attributed to exposure to high ammonia levels, pathogens with the ability to cause conjunctivitis such as *Pasteurella* spp. and *Streptobacillus* spp. were not excluded from the colony or tested for. Exposure to high ammonia levels can also increase susceptibility to pathogens,[4,50,61] so a combination of factors could have been the cause of the observations. We recognize this limitation to the current study; however, evidence of infectious agents was not observed histologically, and similar nasal lesions have been reported in other studies with ammonia inhalation. Furthermore, the lesions in the affected animals had an anterior–posterior distribution, predominantly affecting respiratory epithelium, as well as olfactory epithelium in the dorsal meatus of the nasal cavity, characteristic for an inhaled irritant. In addition, CD1 mice were evaluated in this study to create a worst-case scenario by using a strain with a high number of pups. Other strains and stocks of mice could have different susceptibilities to ammonia. Another limitation was the ammonia monitoring equipment, which only measured levels to 100 ppm, and levels exceeding 100 ppm were recorded as 100 ppm. The 25-ppm threshold used was much lower than this maximum value, so having accurate measurements above 100 ppm was unnecessary but did affect data averages and standard error. Furthermore, the nasal cavities and lungs of adults were not assessed, given that the adults experienced multiple conditions (static and ventilated housing) due to the crossover study design. Age is a factor in

susceptibility to ammonia in humans,[17] but this factor has not been evaluated in mice. Lastly, conditions were evaluated only with 9 pups. This number was chosen in light of our institutional standard operating procedure, which allows for no more than 9 pups past 9 d of age in a trio breeding cage. Other institutions could have trio breeding policies with different husbandry conditions but evaluation was beyond the scope of this study.

In summary, chronic exposure to elevated ammonia levels was associated with a detrimental effect in terms of observed clinical signs and nasal cavity lesions in weanling mice in this study. Given the ammonia levels and histologic findings in our study, it may be necessary to change breeders in static cages more frequently than every 7 d. Ammonia levels in ventilated cages with trio breeders reached 25 ppm by 3 d after cage change, whereas ammonia levels in ventilated cages with paired breeders remained below 25 ppm for 14 d. Given the minimal to mild lesions observed in weanlings from trio-bred ventilated conditions, more frequent cage changes due to elevated ammonia levels may not be necessary. Regardless, additional studies are warranted to evaluate the effects of more frequent cage changes on reproductive parameters, because cage changing is stressful for mice and affects breeding. Overall, research institutions should carefully consider cage change frequency for static caging and high-density breeding scenarios.

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References

1. **Bind RH, Minney SM, Rosenfeld S, Hallock RM.** 2013. The role of pheromonal responses in rodent behavior: future directions for the development of laboratory protocols. J Am Assoc Lab Anim Sci **52:**124–129.
2. **Bolon B, Bonnefoi MS, Roberts KC, Marshall MW, Morgan KT.** 1991. Toxic interactions in the rat nose: pollutants from soiled bedding and methyl bromide. Toxicol Pathol **19:**571–579. https://doi.org/10.1177/019262339101900402.
3. **Braden GC, Rasmussen S, Monette S, Tolwani RJ.** 2017. Effects of breeding configuration on maternal and weanling behavior in laboratory mice. J Am Assoc Lab Anim Sci **56:**369–376.
4. **Broderson JR, Lindsey JR, Crawford JE.** 1976. The role of environmental ammonia in respiratory mycoplasmosis of rats. Am J Pathol **85:**115–130.
5. **Buckley LA, Jiang XZ, James RA, Morgan KT, Barrow CS.** 1984. Respiratory tract lesions induced by sensory irritants at the RD50 concentration. Toxicol Appl Pharmacol **74:**417–429. https://doi.org/10.1016/0041-008X(84)90295-3.
6. **Burn CC, Peters A, Day MJ, Mason GJ.** 2006. Long-term effects of cage-cleaning frequency and bedding type on laboratory rat health, welfare, and handleability: a cross-laboratory study. Lab Anim **40:**353–370. https://doi.org/10.1258/002367706778476460.
7. **Charles River.** [Internet]. 2019. CD-1 IGS Mouse. [Cited 7 May 2019]. Available at: https://www.criver.com/products-services/find-model/cd-1r-igs-mouse?region=3611#.
8. **Corning BF, Lipman NS.** 1991. A comparison of rodent caging systems based on microenvironmental parameters. Lab Anim Sci **41:**498–503.
9. **Crissman JW, Goodman DG, Hildebrandt PK, Maronpot RR, Prater DA, Riley JH, Seaman WJ, Thake DC.** 2004. Best practices guideline: toxicologic histopathology. Toxicol Pathol **32:**126–131. https://doi.org/10.1080/01926230490268756.
10. **Damsch S, Eichenbaum G, Looszova A, Lammens L, Feyen B, Van den Bulck K, Knight E, Kelley M, Tonelli A.** 2010. Unexpected nasal changes in rats related to reflux after gavage dosing. Toxicol Pathol **39:**337–347. https://doi.org/10.1177/0192623310388430.
11. **Danneman PJ, Stein S, Walshaw SO.** 1997. Humane and practical implications of using carbon dioxide mixed with oxygen for anesthesia or euthanasia of rats. Lab Anim Sci **47:**376–385.
12. **De Jonghe S, Lammens L, Raoof A, Steemans K, Broeckaert F, Verbeeck J, Van Goethem F, Hanton G.** 2009. Miscellaneous: P17:Lethal rhinitis/sinusitis in rodents by aspiration of formulation in gavage studies: Importance of evaluation of the nose. Exp Toxicol Pathol **61:**410. https://doi.org/10.1016/j.etp.2009.02.103.
13. **DiVincenti L Jr, Moorman-White D, Bavlov N, Garner M, Wyatt J.** 2012. Effects of housing density on nasal pathology of breeding mice housed in individually ventilated cages. Lab Anim (NY) **41:**68–76. https://doi.org/10.1038/laban0312-68.
14. **Domer DA, Erickson RL, Petty JM, Bergdall VK, Hickman-Davis JM.** 2012. Processing and treatment of corncob bedding affects cage-change frequency for C57BL/6 mice. J Am Assoc Lab Anim Sci **51:**162–169.
15. **Eichenbaum G, Damsch S, Looszova A, Vandenberghe J, Van den Bulck K, Roels K, Megens A, Knight E, Hillsamer V, Feyen B, Kelley MF, Tonelli A, Lammens L.** 2011. Impact of gavage dosing procedure and gastric content on adverse respiratory effects and mortality in rat toxicity studies. J Appl Toxicol **31:**342–354.
16. **Eichner M, Purcell JE, Fortman JD.** 2018. Effects of intracage ammonia on markers of pulmonary endothelial integrity in mice housed in static microisolation cages. J Am Assoc Lab Anim Sci **57:**18–23.
17. **Erskine RJ, Murphy PJ, Langton JA, Smith G.** 1993. Effect of age on the sensitivity of upper airway reflexes. Br J Anaesth **70:**574–575. https://doi.org/10.1093/bja/70.5.574.
18. **Ferrecchia CE, Jensen K, Van Andel R.** 2014. Intracage ammonia levels in static and individually ventilated cages housing C57BL/6 mice on 4 bedding substrates. J Am Assoc Lab Anim Sci **53:**146–151.
19. **Fisher S, Burgess WL, Hines KD, Mason GL, Owiny JR.** 2016. Interstrain differences in CO_2-induced pulmonary hemorrhage in mice. J Am Assoc Lab Anim Sci **55:**811–815.
20. **Gaafar H, Girgis R, Hussein M, el-Nemr F.** 1992. The effect of ammonia on the respiratory nasal mucosa of mice. A histological and histochemical study. Acta Otolaryngol **112:**339–342. https://doi.org/10.1080/00016489.1992.11665429.
21. **Gamble MR, Clough G.** 1976. Ammonia build-up in animal boxes and its effect on rat tracheal epithelium. Lab Anim **10:**93–104. https://doi.org/10.1258/002367776781071477.
22. **Garner JP, Gaskill BN, Pritchett-Corning KR.** 2016. Two of a kind or a full house? Reproductive suppression and alloparenting in laboratory mice. PLoS One **11:**1–13. https://doi.org/10.1371/journal.pone.0154966.
23. **Gaskill BN, Pritchett-Corning KR.** 2015. The effect of cage space on behavior and reproduction in Crl:CD1(Icr) and C57BL/6NCrl laboratory mice. PLoS One **10:**1–22. https://doi.org/10.1371/journal.pone.0127875.
24. **Gautam SH, Rebello MR, Verhagen JV.** 2012. Taste quality and intensity of 100 stimuli as reported by rats: the taste-location association task. Front Behav Neurosci **6:**1–24. https://doi.org/10.3389/fnbeh.2012.00019.
25. **Gonder JC, Laber K.** 2007. A renewed look at laboratory rodent housing and management. ILAR J **48:**29–36. https://doi.org/10.1093/ilar.48.1.29.
26. **Gordon AH, Hart PD, Young MR.** 1980. Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature **286:**79–80. https://doi.org/10.1038/286079a0.
27. **Gordon S, Wallace J, Cook A, Tee RD, Newman Taylor AJ**. 1997. Reduction of exposure to laboratory animal allergens in the workplace. Clin Exp Allergy **27:**744–751. https://doi.org/10.1046/j.1365-2222.1997.640844.x
28. **Green AR, Wathes CM, Demmers TG, Clark JM, Xin H.** 2008. Development and application of a novel environmental preference chamber for assessing responses of laboratory mice to atmospheric ammonia. J Am Assoc Lab Anim Sci **47:**49–56.
29. **Greiner T.** [Internet]. 1995. Indoor air quality: carbon monoxide and carbon dioxide. [Cited 7 May 2019]. Available at: https://www.abe.iastate.edu/extension-and-outreach/indoor-air-quality-carbon-monoxide-and-carbon-dioxide-aen-125/.

30. **Harkema JR.** 1990. Comparative pathology of the nasal mucosa in laboratory animals exposed to inhaled irritants. Environ Health Perspect **85:**231–238.
31. **Harkema JR, Carey SA, Wagner JG.** 2006. The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. Toxicol Pathol **34:**252–269. https://doi.org/10.1080/01926230600713475.
32. **Hasenau JJ, Baggs RB, Kraus AL.** 1993. Microenvironments in microisolation cages using BALB/c and CD-1 mice. Contemp Top Lab Anim Sci **33:**11–16.
33. **Heiderstadt KM, Vandenbergh DJ, Gyekis JP, Blizard DA.** 2014. Communal nesting increases pup growth but has limited effects on adult behavior and neurophysiology in inbred mice. J Am Assoc Lab Anim Sci **53:**152–160.
34. **Hess SE, Rohr S, Dufour BD, Gaskill BN, Pajor EA, Garner JP.** 2008. Home improvement: C57BL/6J mice given more naturalistic nesting materials build better nests. J Am Assoc Lab Anim Sci **47:**25–31.
35. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
36. **Kedl RM, Wysocki LJ, Janssen WJ, Born WK, Rosenbaum MD, Granowski J, Kench JA, Fong DL, Switzer LA, Cruse M, Huang H, Jakubzick CV, Kosmider B, Takeda K, Stranova TJ, Klumm RC, Delgado C, Tummala S, De Langhe S, Cambier J, Haskins K, Lenz LL, Curran-Everett D.** 2014. General parity between trio and pairwise breeding of laboratory mice in static caging. J Immunol **193:**4757–4760. https://doi.org/10.4049/jimmunol.1402306.
37. **Krohn TC, Hansen AK.** 2000. The effects and tolerances for carbon dioxide in relation to recent developments in laboratory animal housing. Scand J Lab Anim Sci **27:**173–181.
38. **Leary SUW, Anthony R, Cartner S, Corey D, Grandin T, Greenacre C, Gwaltney-Brant S, McCrackin MA, Meyer R, Miller D, Shearer J, Yanong R.** 2013. AVMA guidelines for the euthanasia of animals: 2013 ed. [Cited 7 May 2019]. Available at https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.
39. **Lipman NS.** 2007. Design and management of research facilities for mice, p 271–319. In: Fox JG, Davisson MT, Quimby FW, Barthold SW, Newcomer CE, Smith AL, editor. The mouse in biomedical research. Boston (MA): Elsevier. https://doi.org/10.1016/B978-012369454-6/50063-7
40. **Lipman NS.** 1999. Isolator rodent caging systems (State of the art): a critical view. Contemp Top Lab Anim Sci **38:**9–17.
41. **Manninen A, Anttila S, Savolainen H.** 1988. Rat metabolic adaptation to ammonia inhalation. Proc Soc Exp Biol Med **187:**278–281. https://doi.org/10.3181/00379727-187-42664.
42. **Memarzadeh F, Harrison PC, Riskowski GL, Henze T.** 2004. Comparison of environment and mice in static and mechanically ventilated isolator cages with different air velocities and ventilation designs. Contemp Top Lab Anim Sci **43:**14–20.
43. **Mexas AM, Brice AK, Caro AC, Hillanbrand TS, Gaertner DJ.** 2015. Nasal histopathology and intracage ammonia levels in female groups and breeding mice housed in static isolation cages. J Am Assoc Lab Anim Sci **54:**478–486.
44. **Morgan KT.** 1991. Approaches to the identification and recording of nasal lesions in toxicology studies. Toxicol Pathol **19:**337–351. https://doi.org/10.1177/019262339101900 4-103.
45. **National Institute for Occupational Safety and Health.** [Internet]. 2011. Ammonia. Pocket Guide to Chemical Hazards. [Cited 7 May 2019]. Available at: https://www.cdc.gov/niosh/npg/npgd0028.html.
46. **Occupational Safety and Health Administration.** [Internet]. 2012. Occupational Safety and Health Standards. Regulations (Standards – 29 CFR), Table Z-1 Limits of Air Contaminants. [Cited 7 May 2019]. Available at: https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=standards&p_id=9992.
47. **Perkins SE, Lipman NS.** 1995. Characterization and quantification of microenvironmental contaminants in isolator cages with a variety of contact beddings. Contemp Top Lab Anim Sci **34:**93–98.
48. **Perkins SE, Lipman NS.** 1996. Evaluation of microenvironmental conditions and noise generation in three individually ventilated rodent caging systems and static isolator cages. Contemp Top Lab Anim Sci **35:**61–65.
49. **Peters AG, Bywater PM, Festing MF.** 2002. The effect of daily disturbance on the breeding performance of mice. Lab Anim **36:**188–192. https://doi.org/10.1258/0023677021912334.
50. **Pinson DM, Schoeb TR, Lindsey JR, Davis JK.** 1986. Evaluation by scoring and computerized morphometry of lesions of early Mycoplasma pulmonis infection and ammonia exposure in F344/N rats. Vet Pathol **23:**550–555. https://doi.org/10.1177/030098588602300502.
51. **Potgieter FJ, Wilke PI.** 1996. The dust content, dust generation, ammonia production, and absorption properties of three different rodent bedding types. Lab Anim **30:**79–87. https://doi.org/10.1258/002367796780744893.
52. **Rasmussen S, Miller MM, Filipski SB, Tolwani RJ.** 2011. Cage change influences serum corticosterone and anxiety-like behaviors in the mouse. J Am Assoc Lab Anim Sci **50:**479–483.
53. **Reeb-Whitaker CK, Harrison DJ, Jones RB, Kacergis JB, Myers DD, Paigen B.** 1999. Control strategies for aeroallergens in an animal facility. J Allergy Clin Immunol **103:**139–146. https://doi.org/10.1016/S0091-6749(99)70537-3.
54. **Reeb-Whitaker CK, Paigen B, Beamer WG, Bronson RT, Churchill GA, Schweitzer IB, Myers DD.** 2001. The impact of reduced frequency of cage changes on the health of mice housed in ventilated cages. Lab Anim **35:**58–73. https://doi.org/10.1258/0023677011911381.
55. **Reeb C, Jones R, Bearg D, Bedigan H, Myers D, Paigen B.** 1998. Microenvironment in ventilated animal cages with differing ventilation rates, mice populations, and frequency of bedding changes. Contemp Top Lab Anim Sci **37:**43–49.
56. **Reeb CK, Jones RB, Bearg DW, Bedigian H, Paigen B.** 1997. Impact of room ventilation rates on mouse cage ventilation and microenvironment. Contemp Top Lab Anim Sci **36:**74–79.
57. **Renne R, Brix A, Harkema J, Herbert R, Kittel B, Lewis D, March T, Nagano K, Pino M, Rittinghausen S, Rosenbruch M, Tellier P, Wohrmann T.** 2009. Proliferative and nonproliferative lesions of the rat and mouse respiratory tract. Toxicol Pathol **37 7_Suppl:**5S–73S. https://doi.org/10.1177/0192623309353423.
58. **Rahija RJ.** 2007. Gnotobiotics, p 217–233. In: Fox JG, Davisson MT, Quimby FW, Barthold SW, Newcomer CE, Smith AL, editor. The mouse in biomedical research. Boston (MA): Academic Press.
59. **Rosenbaum MD, VandeWoude S, Johnson TE.** 2009. Effects of cage-change frequency and bedding volume on mice and their microenvironment. J Am Assoc Lab Anim Sci **48:**763–773.
60. **Ruehl-Fehlert C, Kittel B, Morawietz G, Deslex P, Keenan C, Mahrt CR, Nolte T, Robinson M, Stuart BP, Deschl U, RITA Group; NACAD Group.** 2003. Revised guides for organ sampling and trimming in rats and mice–part 1. Exp Toxicol Pathol **55:**91–106. https://doi.org/10.1078/0940-2993-00311.
61. **Saito M, Nakayama K, Muto T, Nakagawa M.** 1982. Effects of gaseous ammonia on *Mycoplasma pulmonis* infection in mice and rats. Jikken Dobutsu **31:**203–206.
62. **Serrano LJ.** 1971. Carbon dioxide and ammonia in mouse cages: effect of cage covers, population, and activity. Lab Anim Sci **21:**75–85.
63. **Silverman J, Bays DW, Baker SP.** 2009. Ammonia and carbon dioxide concentrations in disposable and reusable static mouse cages. Lab Anim (NY) **38:**16–23. https://doi.org/10.1038/laban0109-16.
64. **Silverman J, Bays DW, Cooper SF, Baker SP.** 2008. Ammonia and carbon dioxide concentrations in disposable and reusable ventilated mouse cages. J Am Assoc Lab Anim Sci **47:**57–62.
65. **Solomon NG, Keane B.** 2007. Reproductive strategies in female rodents, p 42–56. In: Wolff JO, Sherman PW, editors. Rodent societies: an ecological and evolutionary perspective. Chicago (IL): University of Chicago Press
66. **Speakman JR, Keijer J.** 2012. Not so hot: optimal housing temperatures for mice to mimic the thermal environment of humans. Mol Metab **2:**5–9. https://doi.org/10.1016/j.molmet.2012.10.002.
67. **Studier CH, Baca TP.** 1968. Atmospheric conditions in artificial rodent burrows. Southwest Nat **13:**401–410. https://doi.org/10.2307/3668907.

68. **Svenson KL, Paigen B.** 2019. Recommended housing densities for research mice: filling the gap in data-driven alternatives. FASEB J **33:**3097–3111. https://doi.org/10.1096/fj.201801972R.
69. **Tsai PP, Oppermann D, Stelzer HD, Mahler M, Hackbarth H.** 2003. The effects of different rack systems on the breeding performance of DBA/2 mice. Lab Anim **37:**44–53. https://doi.org/10.1258/002367703762226683.
70. **Van Loo PL, Van Zutphen LF, Baumans V.** 2003. Male management: coping with aggression problems in male laboratory mice. Lab Anim **37:**300–313. https://doi.org/10.1258/002367703322389870.
71. **Vogelweid CM, Zapien KA, Honigford MJ, Li L, Li H, Marshall H.** 2011. Effects of a 28-day cage-change interval on intracage ammonia levels, nasal histology, and perceived welfare of CD1 mice. J Am Assoc Lab Anim Sci **50:**868–878.
72. **Washington IM, Payton ME.** 2016. Ammonia levels and urine-spot characteristics as cage-change indicators for high-density individually ventilated mouse cages. J Am Assoc Lab Anim Sci **55:**260–267.
73. **Wasson K.** 2017. Retrospective analysis of reproductive performance of pair-bred compared with trio-bred mice. J Am Assoc Lab Anim Sci **56:**190–193.