The impact of reduced frequency of cage changes on the health of mice housed in ventilated cages

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Summary

Our purpose in this investigation was to determine if we could reduce cage changing frequency without adversely affecting the health of mice. We housed mice at three different cage changing frequencies: 7, 14, and 21 days, each at three different cage ventilation rates: 30, 60 and 100 air changes per hour (ACH), for a total of nine experimental conditions. For each condition, we evaluated the health of 12 breeding pairs and 12 breeding trios of C57BL/6J mice for 7 months. Health was assessed by breeding performance, weanling weight and growth, plasma corticosterone levels, immune function, and histological examination of selected organs. Over a period of 4 months, we monitored the cage microenvironment for ammonia and carbon dioxide concentrations, relative humidity, and temperature one day prior to changing the cage. The relative humidity, carbon dioxide concentrations, and temperature of the cages at all conditions were within acceptable levels. Ammonia concentrations remained below 25 ppm (parts per million) in most cages, but, even at higher concentrations, did not adversely affect the health of mice. Frequency of cage changing had only one significant effect; pup mortality with pair matings was greater at the cage changing frequency of 7 days compared with 14 or 21 days. In addition, pup mortality with pair matings was higher at 30 ACH compared with other ventilation rates. In conclusion, under the conditions of this study, cage changes once every 14 days and ventilation rates of 60 ACH provide optimum conditions for animal health and practical husbandry.

Keywords Cage changing frequency; animal health; cage microenvironment; ammonia; ventilated caging; animal husbandry; reproductive performance; growth rate

Over the past decade, technology for housing laboratory animals has continued to evolve. Initially, to protect mice from infections, filter covers were introduced on static cages. Since then, to further improve the health of both workers and mice, a variety of cage ventilation systems have been developed and have become widely accepted. Although the cage microenvironment is documented in some of these systems (Wu *et al*. 1985, Corning & Lipman 1991, Huerkamp *et al*. 1994, Huerkamp & Lehner 1994, Clough *et al*. 1995, Perkins & Lipman 1996, Tu *et al*. 1997, Reeb *et al*. 1998), cage ventilation rates and cage changing frequencies that are best for animal health are not well understood.

In the 1970s, The Jackson Laboratory (Bar Harbor, ME), in cooperation with Thoren Caging Systems (Hazleton, PA, USA), developed an individually ventilated cage system. Whereas the average ventilation rate in static cages in a room ventilated at 10–20 ACH was

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16–19 ACH (Reeb *et al*. 1997), the ventilated system can be adjusted from 30 to 100 ACH. Although early studies of the ventilated caging system indicated that bedding in ventilated cages remains dry even when changed less frequently than static cages (E. P. Les, personal communication), these observations were not pursued in detail. It is still standard procedure at The Jackson Laboratory to change ventilated cages every 7 days, the same frequency at which static cages are changed. 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 (Gamble & Clough 1976, Serrano 1971). The facts that ventilated cages are drier and that ventilation rates can be altered in mechanically-ventilated cages raise the possibility that mouse cages can be changed less frequently without adversely affecting animal health.

The *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996) states that mouse cages must usually be sanitized weekly, but indicates that ventilated cages might require less frequent sanitization without making any specific recommendations in this regard. Rather, it suggests that the frequency be determined by professional judgement. Yet, there is little information upon which to make such a judgement in automatically-ventilated cages. We sought to better understand the cage microenvironment, to improve health conditions for both the mice and facility technicians, and to reduce the costs of managing the research animal facility.

Our purpose in this investigation was to determine if we could reduce cage changing frequency without adversely affecting animal health. We also wanted to identify the cage ventilation that is optimal for the health of mice. Knowing the optimal ventilation rate will assist in the design of cost efficient equipment. We experimented with three different cage changing frequencies: every 7, 14 and 21 days, each at three different cage ventilation rates of 30, 60 and 100 ACH, for a total of nine experimental conditions. For 7 months, we evaluated the health of 12 breeding pairs and 12 breeding trios (two females, one male) of C57BL/6J mice and their cage microenvironments for each of the nine conditions. The health of mice was assessed by their breeding performance, weanling weight and growth, plasma corticosterone levels, and a necropsy followed by histological examination of key organs.

Our long-range goal is to better understand the ecosystem of the laboratory mouse and to provide a healthier environment for both mice and humans at the lowest possible cost. This goal is especially relevant in a world where health and safety standards in the workplace are more demanding than ever, and the cost of research rises and competes with other societal needs.

Materials and methods

Animals

C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were maintained in the closed colonies of the research animal facility of The Jackson Laboratory. The mice were obtained from colonies that are systematically monitored and negative for well known agents including 17 viruses, 10 bacteria, 2 *Mycoplasma* spp., 6 protozoa, and 12 parasites. Details are available at http://jaxmice.jax.org. The mice were either trio-mated (two females, one male) or pair-mated (one female, one male) at 4 weeks of age and bred through 34 weeks of age. Pup density was unrestricted for pups less than 14 days of age and then reduced to a maximum of 12 older pups per cage. Mice were housed in duplex (two pens) polycarbonate cages, $31 \times 31 \times 14$ cm, and each pen was covered by a wire-rod metal top holding feed pellets and a water bottle. All ventilated cages were covered by a snap-on filter top (Reemay 2033, Thoren Caging Systems Inc., Hazleton, PA, USA) and static (non-ventilated) control cages were covered by a filter bonnet top. Mice had *ad libitum* access to acidified bottled water (pH 2.8–3.1) and pelleted feed (diet D-11, Purina Mills Inc., Richmond, IN, USA). Autoclaved white pine shavings (Crobb Box Co., Ellsworth, ME, USA) were used as bedding. The light cycle was 14 h light and 10 h

dark. At the conclusion of the experiments, animals were euthanazed with carbon dioxide gas. Our animal facilities and animal care and use programmes are accredited by the Association for the Accreditation of Laboratory Animal Care International and the experiments were approved by the Institutional Animal Care and Use Committee.

Animal room and caging systems

The 5×12.8 m mouse room was supplied with high efficiency particulate arresting (HEPA) filtered air at 15 ± 1 ACH and maintained at $45 \pm 5\%$ relative humidity and $22 \pm 2°C$ temperature. Experimental mice were housed in 3 mobile racks, each with a capacity of 64 individually-ventilated cages (Maxi-Miser™ PIV; Thoren Caging Systems, Inc., Hazleton, PA, USA). The ventilated cage system circulated room air through a HEPA filter and into the animal cages. Cage exhaust air was circulated through a second HEPA filter before being discharged back into the room. Cage air pressure was positive with respect to the room. A comparison group of mice were housed nearby on a static (non-ventilated) rack. In addition to these experiments, the room contained 19 non-ventilated animal racks and had a total working census of approximately 615 cages.

Cage ventilation rates We adjusted the cage ventilation rates with dampers in the supply and exhaust ducts of the racks. Cage air exchange rates were measured using sulphur hexaflouride tracer gas, a multi-point tracer gas doser/air sampler, and gas analyser (models 1302 and 1303, Brüel and Kjær, Nærum, Denmark). Detailed methods for calculating cage air exchange rates are published elsewhere (Reeb *et al*. 1998). We previously determined that all cages within our ventilated rack system received equal amounts of ventilation (Reeb *et al*. 1998).

Effect of cage ventilation and cage changing frequency We experimented with three different cage ventilation rates: 30, 60 and 100 ACH, each in separate racks. Within each rack, we used three cage changing frequencies, every 7, 14 and 21 days, for a total of nine experimental conditions (see Fig 1). Each of the nine experimental conditions had 24 breeding groups, 12 trio-mated and 12 pair-mated groups. The experiment with breeding pairs was initiated after the study with breeding trios was completed; both

Rack 1 Cage ventilation: 30 ACH*	Rack 2 Cage ventilation: 60 ACH	Rack 3 Cage ventilation: 100 ACH	Rack 4 Control Static
7 day change	7 day change	7 day change	7 day change
14 day change	14 day change	14 day change	------
21 day change	21 day change	21 day change	------

Fig 1 Experimental design. Experiment 1 evaluated the health effects of 12 *trio*-mated breeding groups housed in each of the nine experimental conditions and one control condition. Three racks, ventilated at 30, 60 and 100 air changes/h (ACH) contained cages changed at a frequency of once every 7, 14, or 21 days. One static rack was established as a comparison rack, and contained cages changed once every 7 days. Experiment 2 was initiated after Experiment 1 was completed, and tested the health of 12 pair-mated breeding groups at each condition illustrated above. *ACH = number of air changes per hour

studies utilized the same racks and were carried out under basically identical conditions. For the comparison groups in unventilated cages, 12 pair or trio-mated mice were housed in static duplex cages that were changed once every 7 days. Because ammonia concentrations greater than 100 ppm were observed in cages changed every 21 days at 30 ACH, all breeding trios housed at 30 ACH were terminated after the birth of the first litter because of concerns that these ammonia levels might harm the mice.

Cage microenvironment To assess the cage microenvironment, we set up three additional cages with breeding mice for each of the nine groups. We did this to avoid disturbing cages of mice under observation for breeding performance and health status. Using the multi-point gas analyser described above, we measured temperature and concentrations of ammonia, carbon dioxide, and water vapour in the cages and the room over a 4-month period for both pair and trio-mated populations. Measurements were taken three times between 13:00 and 17:00 h on days 6, 13, or 20 of soiled bedding. At the time of monitoring, there were 7 to 12 pups per cage with trio-mated breeders and 6 to 11 pups per cage with pair-mated breeders. We sampled cage air through two fittings drilled in the front and 5 cm from the bottom of each cage. Measurement error was $\pm 2.5\%$ for gases and water vapour and $\pm 0.2°C$ for temperature. We calculated per cent relative humidity from water vapour concentration and temperature.

Animal health assessment

Fecundity To evaluate the reproductive success of each female in the study, we recorded the number of pups born, the number of pups weaned, pup mortality, average number of litters, average litter size at birth, breeder mortality and females that failed to breed.

Histology For each of the nine experimental conditions and comparison groups, we necropsied eight breeders (four females, four males) when they were approximately 8 months old. Kidney, liver, gall bladder, adrenal glands, lung, trachea and gonads were removed, fixed in Bouin's solution, routinely processed and imbedded in paraffin. The organs were sectioned at 5 μm, stained with haematoxylin and eosin, and evaluated.

To further investigate the effect that fluctuating naturally developing ammonia concentrations have on nasal passages of mice, 60 trio breeding units were set up in static cages changed once every 14 days. We used static cages in order to obtain higher ammonia concentrations. The cycle of 14-day changing was established to coincide with litter birth, age 14 days, and age 28 days when the animals were ready to be weaned. From birth to weaning, ammonia concentrations were measured weekly on the day before the cages were scheduled to be changed. The ammonia levels were highest at the time of weaning, and these levels determined into which exposure range mice were placed: less than 25 ppm, 25–50 ppm, 50–75 ppm, 75–100 ppm, and greater than 100 ppm. We planned to use the ammonia concentrations that occurred naturally, but this failed to yield enough mice in the lower and higher ammonia concentration groups. To attain the lower targeted ammonia concentrations, pups were culled in some cages. To attain the higher targeted concentrations, we had to add 5–10 ml of water beginning at day 13 after birth and repeated on Mondays, Wednesdays and Fridays until day 28 to increase intracage humidity and ammonia levels. At all targeted ranges, 20 breeding adults (10 females, 10 males) at 16 weeks of age and 10 weanlings at 4 weeks of age were examined. Mice were euthanazed and heads were removed, fixed and decalcified, processed, sectioned, and stained as described above for other organs. Transverse sections were taken through the nasal passages at five locations: the anterior base of the incisors, the posterior base of the incisors, the incisive papilla, the second palatine ridge, and the second molar teeth.

Weanling body weight Weights of 40 mice (20 of each sex) from each group were recorded when they were 28 ± 3 days old. Mice were weighed at the same time of day between 07:00 and 09:00 h.

Growth From ages 4 to 11 weeks, 16 weanlings (eight females, eight males) from each experimental and control group were weighed weekly between 07:00 and 09:00 h. These weanlings were kept in groups of four of the same sex, at the same cage conditions in which they were born.

Plasma corticosterone concentrations We measured plasma corticosterone concentrations of 4-week-old mice that had been placed in eight groups of four of the same sex and housed at the same conditions in which they were born. Other than for cage changing, these mice were only disturbed when we sampled blood at 13 weeks of age. The blood sample was obtained from the retro-orbital sinus without anaesthesia by an experienced technician. Because plasma corticosterone is very labile, we took precautions to reduce animal stress during sample collection. We sampled only one mouse per cage and obtained the blood sample within 60 s of removing the mouse from the cage. We sampled them between 08:00 and 10:00 h on Sunday mornings when animal rooms were quiet. Plasma corticosterone was measured by an I^{125} double-antibody radioimmunoassay (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA).

Immune function To measure the long-term effect of a reduced changing schedule on leucocyte profiles, eight pens containing four male C57BL/6J mice each were maintained in the same Thoren PIV rack at 60 ACH for 5 months. Four pens were changed every 7 days, and four pens were changed every 14 days. The cage microenvironment was monitored on the day before the cage was changed for ammonia, water vapour, carbon dioxide levels and temperature. At the end of 5 months, we collected blood from four mice, one per cage, of each group for flourescence activated cell sorting (FACS). Peripheral blood leucocytes (PBL) and splenocytes were lysed with ACK lysing buffer (Coligan *et al.* 1998), and washed with FACS buffer (CMF-PBS + 1% BSA + 0.05% NaN3) prior to staining. Cells were blocked with a combination of 10 μg rat IgG (Sigma Chemical, St Louis, MO, USA) and 10 μg clone 24G.2 (anti CD16/32[FcgII/IIIRc]) (The Jackson Laboratory, Bar Harbor, ME, USA) for 30 min at room temperature prior to staining. Fifty microlitres of cells at a concentration of 2×10^7 cells/ml (10^6 cells) were added to 10 μl of antibody cocktail. Monoclonal antibody clones 53–6.72 (anti CD8a) PE, M1/70 (anti CD11b/Mac-1) PE, and RA3-6B2(anti CD45R/B220) PE were obtained from PharMingen (San Diego, CA, USA). F(ab′)2amuIg (A,D,G,M) fluorescein isothiocynate (FITC) was obtained from Southern Biotech (Fisher Scientific, Pittsburgh, PA, USA). Clones 145-2C11 (anti CD3e), GK1.5 (anti CD4), and RB6-8C5 (anti Ly6G/Gr-1) were obtained as hybridomas from various sources and are maintained at The Jackson Laboratory (Bar Harbor, ME, USA). Hybridomas were expanded in culture, the culture supernatant precipitated with 45% saturated ammonium sulphate, dialyzed against calcium/magnesium-free phosphate buffered saline and 0.05% sodium azide. The equilibrated antibody mixture was further purified by gel filtration over a 2.5/100 AcA22 column at a rate of 6 ml/h at room temperature. The Ig fraction was pooled for further conjugation. 145-2C11 and RB6-8C5 antibodies were conjugated with (FITC) as described (Harlow *et al.* 1988). GK1.5 was conjugated with Cy3 using the Fluoro-link kit (Amersham Pharmacia Biotech, Inc, Piscataway, NJ, USA). All antibodies were titrated by flow cytometry to optimize staining and resolution. The cells were incubated with the antibodies for 30 min at 4°C and washed with 2 ml FACS buffer. The cells were pelleted at 500 g, the supernatant decanted, and re-suspended in 500 μl of FACS buffer. All experiments were performed on the FACScan cytometer (Becton Dickinson, San Jose, CA, USA). Acquisition and analysis were performed using Cellquest software.

Statistical analysis

Values are reported as mean ± SEM. The statistical significance of data for reproductive performance, cage microenvironment, and most health parameters, was determined by analyses of variance (ANOVA, $P < 0.05$) followed by Tukey *post hoc* tests of the differences in means (Tables 1, 2, 3, 5). No

confounding effects were found between cage ventilation rates and cage changing frequency, but confounding effect was observed between pair-mated and trio-mated populations so these groups were analysed separately. To take into account the age of the mice, we performed an analysis of co-variance (ANCOVA, $P < 0.05$) with age as a covariate (Table 4). Breeder mortality and productivity were analysed using Fisher's exact test ($P < 0.05$).

Results

Cage microenvironment We previously published the microenvironment data of trio-mated populations (Reeb *et al*. 1998); the results of the microenvironment assessment for pair-mated populations are shown here (Table 1, Figs 2a and 2b). Microenvironment parameters were generally lower in pair-mated populations than in trio-mated populations.

Table 1 Effect of cage ventilation and frequency of cage change on the microenvironment of pair-mated breeding populations

	Frequency of cage change, days [mean ± SEM (*n*)*]		
	7	14	21
Ammonia (ppm)			
30 air changes/h	26.3 ± 5.7[‡a] (12)	62.8 ± 17.6[a] (5)	73.0 ± 15.4[a] (4)
60 air changes/h	1.5 ± 0.2[‡b] (14)	14.6 ± 6.7[b] (10)	26.9 ± 19.1[b] (9)
100 air changes/h	1.1 ± 0.2[§b] (13)	3.7 ± 1.5[b] (8)	15.4 ± 7.4[b] (6)
Relative humidity (%)			
30 air changes/h	57 ± 1[a]	52 ± 2	57 ± 4
60 air changes/h	48 ± 2[b]	53 ± 4	52 ± 3
100 air changes/h	48 ± 2[b]	51 ± 3	46 ± 2
Carbon dioxide (ppm)			
30 air changes/h	2190 ± 185[a]	1475 ± 90	2050 ± 215[a]
60 air changes/h	1310 ± 145[b]	1775 ± 300	1415 ± 240[a,b]
100 air changes/h	1110 ± 110[b]	1575 ± 270	945 ± 200[b]
Temperature (°C)			
30 air changes/h	24.4 ± 0.2	24.4 ± 0.3	24.8 ± 0.6
60 air changes/h	24.1 ± 0.4	24.1 ± 0.6	23.4 ± 0.5
100 air changes/h	23.2 ± 0.3	23.2 ± 0.2	24.1 ± 0.5

*(*n*) is the number of measurements and applies to all parameters; [‡] Value is significantly different from 14 and 21 day conditions in same row ($P < 0.05$); [§] Value is significantly different from 21 day condition in same row ($P < 0.05$); [a] Within a column, values with different superscript letters differ significantly ($P < 0.05$)

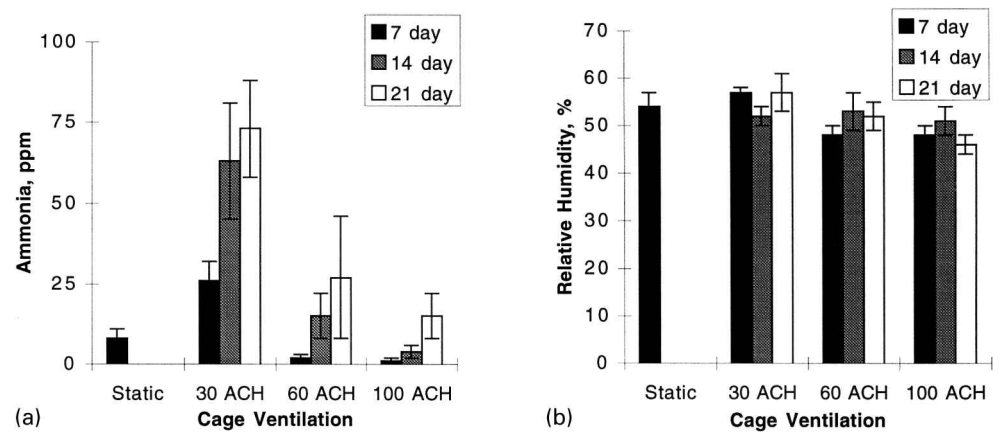

Fig 2 Cage microenvironment. Ammonia (a) and relative humidity (b) concentrations in ventilated cages housing pair-mated breeders and their pups. Cages were either static (comparison) or ventilated at 30, 60 or 100 air changes/h. Frequency of cage change was once every 7, 14, or 21 days

Ammonia Ammonia concentrations as measured on the day before cage changing were consistently lower at higher ventilation rates. In cages housing pair-mated mice, mean ammonia concentrations were approximately 25 ppm or less at 60 and 100 ACH, even with reduced cage changing frequencies.

In cages housing trio-mated mice, mean ammonia concentrations were less than 25 ppm at all ACH when cages were changed once every 7 days and at 100 ACH when cages were changed every 14 days. Mean ammonia concentrations averaged approximately 50 ppm at 30 and 60 ACH and 25 ppm at 100 ACH when cages were changed once every 14 days. Ammonia concentrations averaged approximately 135 ppm, 125 ppm, and 45 ppm at 30, 60 and 100 ACH respectively when cages were changed once every 21 days (Reeb *et al.* 1998).

Relative humidity None of the relative humidity measurements were significantly different from the values observed in the static comparison cages. With pair-mated mice, relative humidity was significantly higher at 30 ACH than at 60 or 100 ACH when cages were changed every 7 days. Within a given ventilation rate for both pair and trio-mated mice, relative humidity did not differ significantly among the cage changing frequencies, suggesting that forced ventilation is able to prevent a build-up of moisture inside the cage.

Carbon dioxide Carbon dioxide concentrations tended to decrease with increasing ventilation. Carbon dioxide ranged from approximately 945–2200 ppm in pair-mated mice (Table 1) and from 1400–3660 ppm in trio-mated mice (Reeb *et al.* 1998).

Temperature Temperature in pair-mated cages also tended to decrease with increased ventilation, however temperature in cages housing trio-mated animals had no clear trends. Temperature ranged from 24.1–24.8°C in pair-mated populations (Table 1) and from 24.0–26.1°C in trio-mated populations (Reeb *et al.* 1998).

Room environment In the room environment ammonia concentrations ranged from 0.9–1.3 ppm, humidity ranged from 41–48%, carbon dioxide ranged from 480–520 ppm, and temperature ranged from 20.9–22.2°C.

Animal heath assessment

Fecundity The effects that cage ventilation rates and cage change frequencies had on fecundity are summarized in Tables 2 and 3. Pup mortality in breeding pairs was 3.4 ± 0.7 in cages changed every 7 days, 1.3 ± 0.4 in cages changed every 14 days, and 1.6 ± 0.5 every 21 days, suggesting that reduced changing frequency was beneficial to pup survival. The difference in pup mortality between 7 and 14-day cage changing was statistically significant. The same benefit was observed for breeding trios, but the difference between 7 and 14 days did not reach statistical significance (5.9 ± 0.7 versus 4.3 ± 0.6 pups respectively). The lowest ventilation rate had an adverse effect on pup mortality. For breeding pairs, pup mortality was 3.3 pups/dam at 30 ACH, significantly greater than the mortality rates of 1.8 pups/dam and 1.2 pups/dam at 60 and 100 ACH respectively. (A similar comparison could not be made for breeding trios because breeding trios at 30 ACH were discontinued after the first litter).

Breeder mortality The average breeding female mortality (4%) and the average number of females that did not produce a litter (< 3%) were comparable with the historical productivity of C57BL/6J mice at The Jackson Laboratory (Fox & Witham 1997) and were not affected by ventilation rate or cage changing frequency for either breeding pairs or trios.

Histology In both retired breeding pairs and trios, no lesions were observed in the selected organs examined that correlated with any combination of cage ventilation rate and cage changing frequency. We examined eight 8-month-old breeders (four female, four male) from cage changing frequencies of 7 and 21 days at all ventilation rates. In 67 of 72 mice from breeding pairs and 46 of 56 mice from breeding trios, we found mild inflammatory

Table 2 Breeding fecundity of pair-mated and trio-mated mice

	Mice born per dam mean ± SE		Mice weaned per dam mean ± SE		Pre-wean mortality No. pups/dam mean ± SE		Wean-to-born ratio (%)		Litters born per dam mean ± SE		Mice born per litter mean ± SE	
	Pair	Trio	Pair	Trio	Pair	Trio	Pair	Trio	Pair	Trio	Pair	Trio
Comparison	22.3 ± 2.9 (10)	21.2 ± 1.1 (18)	20.7 ± 2.4	16.8 ± 1.3	1.6 ± 0.9	4.4 ± 1.0	92.8	79.2	3.5 ± 0.4	3.9 ± 0.2	6.6 ± 0.4	5.9 ± 0.4
Cage ventilation												
30 ACH	21.0 ± 1.3 (35)	–	17.8 ± 1.2[a]	–	3.3 ± 0.8*[,a]	–	84.8	–	3.3 ± 0.2	–	6.6 ± 0.2	–
60 ACH	24.9 ± 1.5 (35)	23.8 ± 1.0 (72)	23.1 ± 1.3[b]	17.7 ± 0.9	1.8 ± 0.5[b]	5.4 ± 0.6	92.8	74.4	3.6 ± 0.2	4.0 ± 0.2	7.0 ± 0.2	6.1 ± 0.2
100 ACH	20.8 ± 1.6 (34)	23.9 ± 1.1 (71)	19.6 ± 1.5[a,b]	18.4 ± 0.9	1.2 ± 0.5[b]	4.6 ± 0.5	94.2	77.0	3.1 ± 0.2	3.8 ± 0.2	6.9 ± 0.3	6.3 ± 0.2
Frequency of cage change												
7 day	24.0 ± 1.5 (35)	25.1 ± 1.4 (47)	20.6 ± 1.3	18.7 ± 1.2	3.4 ± 0.7[a]	5.9 ± 0.7	85.8	74.5	3.5 ± 0.2	3.9 ± 0.2	7.1 ± 0.2	6.4 ± 0.2
14 day	21.3 ± 1.5 (35)	24.4 ± 1.0 (48)	20.0 ± 1.4	19.2 ± 0.9	1.3 ± 0.4[b]	4.3 ± 0.6	93.9	78.7	3.2 ± 0.2	4.2 ± 0.2	6.8 ± 0.3	5.9 ± 0.2
21 day	21.5 ± 1.6 (34)	22.0 ± 1.6 (48)	19.9 ± 1.5	16.3 ± 1.2	1.6 ± 0.5[a,b]	4.8 ± 0.8	92.6	74.1	3.4 ± 0.2	3.5 ± 0.2	6.7 ± 0.3	6.3 ± 0.2

[a] Within the experimental conditions of ventilation and frequency of cages change, values within columns having different superscripts are significantly different (ANOVA, $P < 0.05$); * Value is different from comparison; (*n*) number of productive female mice; ACH = number of air changes per hour. Although all conditions were evaluated separately in the initial analysis, for ease of presentation, the ventilation rates are compared using all data from the different cage change frequencies. Likewise, the cage changing frequencies are compared using all data from the three ventilation rates. This was valid because there was no confounding for cage ventilation and cage changing frequency

Table 3 Pre-wean mortality by cage ventilation rate

	Pre-wean mortality, No. pups/dam mean ± SE	
	Pair (*n*)	Trio (*n*)
Comparison	1.6 ± 0.9 (10)	4.4 ± 1.0 (18)
30 ACH		
7 day	4.8 ± 1.3*[,a] (12)	–
14 day	1.8 ± 0.6[b] (12)	–
21 day	3.1 ± 0.7[a,b] (11)	–
60 ACH		
7 day	3.3 ± 1.2[a] (12)	5.8 ± 1.8 (24)
14 day	1.9 ± 0.9[a,b] (11)	4.8 ± 1.0 (24)
21 day	0.3 ± 0.1[b] (12)	5.7 ± 1.3 (24)
100 ACH		
7 day	2.0 ± 0.9 (11)	6.1 ± 1.0 (23)
14 day	0.2 ± 0.1 (12)	3.7 ± 0.8 (24)
21 day	1.5 ± 1.1 (11)	4.0 ± 0.9 (24)

[a] Within the experimental conditions of ventilation and frequency of cages change, values within columns having different superscripts are significantly different (ANOVA, $P < 0.05$); * Value is different from comparison; (*n*) number of productive female mice; ACH = number of air changes per hour

or degenerative lesions typical of older mice. Most commonly found were chronic interstitial nephritis and hepatic lipidosis; other changes also observed were mild and had no identifiable causes. Changes in the adrenal glands that have been associated with chronic stress were not observed.

We observed no differences in nasal passage histology among the five ammonia exposure concentrations of <25 ppm, 25–50 ppm, 50–75 ppm, 75–100 ppm, and >100 ppm (Table 6). Nearly all mice had a small amount of proteinaceous secretion on the mucosal surface (Fig 3B), and about 20% of them had scattered groups of 1–35 inflammatory cells, mostly lymphocytes, in the lumen and occasionally in the submucosa (Fig 3B). However, neither the percentage of mice with inflammatory cells nor the number of inflammatory cells was related to the ammonia exposure level. Though four mice had a small abcess or ulcer (Fig 3C), these were not related to ammonia exposure since they occurred in both the lowest and highest ammonia concentrations. The only significant inflammation we observed was associated with foreign bodies such as shavings or hair.

Table 4 Body weight of pups at time of weaning, 4 weeks of age

	Body weight of weanling mice (g ± SEM)			
	Offspring from pair-mated breeders		Offspring from trio-mated breeders	
	Female (*n*)*	Male	Female (*n*)	Male
Comparison (static cages)	14.7 ± 0.7 (16)	15.7 ± 0.4 (15)	15.3 ± 0.4 (10)	16.5 ± 0.3 (16)
Cage ventilation				
30 ACH	15.1 ± 0.8[a] (60)	15.6 ± 0.3[a]	–	–
60 ACH	14.7 ± 0.2[a,b] (60)	16.6 ± 0.3[b]	15.7 ± 0.2 (60)	16.6 ± 0.2
100 ACH	14.0 ± 0.2[b] (60)	14.7 ± 0.2[c]	15.6 ± 0.3 (60)	17.0 ± 0.3
Frequency of cage change				
7 day	14.6 ± 0.2 (60)	15.7 ± 0.2	15.5 ± 0.3 (40)	16.8 ± 0.4
14 day	15.0 ± 0.3 (60)	15.8 ± 0.2	16.2 ± 0.2 (40)	16.6 ± 0.3
21 day	14.3 ± 0.3 (60)	15.4 ± 0.4	15.2 ± 0.3 (40)	17.0 ± 0.3

*The number of males is that listed under females for each breeding type; [a] Within ventilation of frequency of cage change conditions, values within columns having different superscripts are significantly different (ANCOVA, $P < 0.05$). Age used as covariate. ACH = number of air changes per hour

Weanling body weight We observed no significant differences among the weights of weanlings born from trio matings for any of the different ventilation rates or cage changing frequencies (Table 4). Similarly, we observed no significant differences among the weights of weanlings born from pair matings for any of the different cage changing frequencies (Table 4). However, weanlings born from breeding pairs weighed significantly less if housed at 100 ACH than if housed at 30 ACH with female weanlings and at both 30 and 60 ACH with male weanlings (Table 4).

Growth Growth curves for 7 weeks after weaning of all offspring in all husbandry conditions were similar. Because the curves were similar, Figs 4a and 4b show only those for offspring housed either in static cages or at 60 ACH with cage changing frequencies of 7 and 21 days.

Plasma corticosterone concentrations Corticosterone concentrations in all conditions were less than 50 ng/ml of plasma, a concentration similar to measurements of 'control' mice previously published (Levine & Treiman 1964, Beamer *et al.* 1994), and consistent with non-stressful conditions. Though not a statistically significant trend, corticosterone levels tended to be lower at lower cage changing frequencies (Table 5).

Table 5 Adult plasma corticosterone levels

	Corticosterone levels ng/ml ± SEM Breeder
Comparison (static)	22 ± 7 (8)
30 ACH	
7 day	46 ± 13 (8)
14 day	36 ± 12 (8)
21 day	29 ± 7 (8)
60 ACH	
7 day	46 ± 10 (8)
14 day	45 ± 14 (8)
21 day	32 ± 12 (8)
100 ACH	
7 day	38 ± 14 (8)
14 day	25 ± 14 (8)
21 day	20 ± 7 (8)

No significant difference within cage ventilation rates (ANCOVA, $P > 0.05$). ACH = number of air changes per hour

Immune function Results from the FACSCAN evaluation of white blood cells showed no significant difference in percentage of cells present between mice changed every 7 days and those changed every 14 days (Table 7).

Discussion

Our investigation has demonstrated that reducing the frequency of cage changing in our ventilated system from once every 7 days to once every 14 or 21 days did not adversely

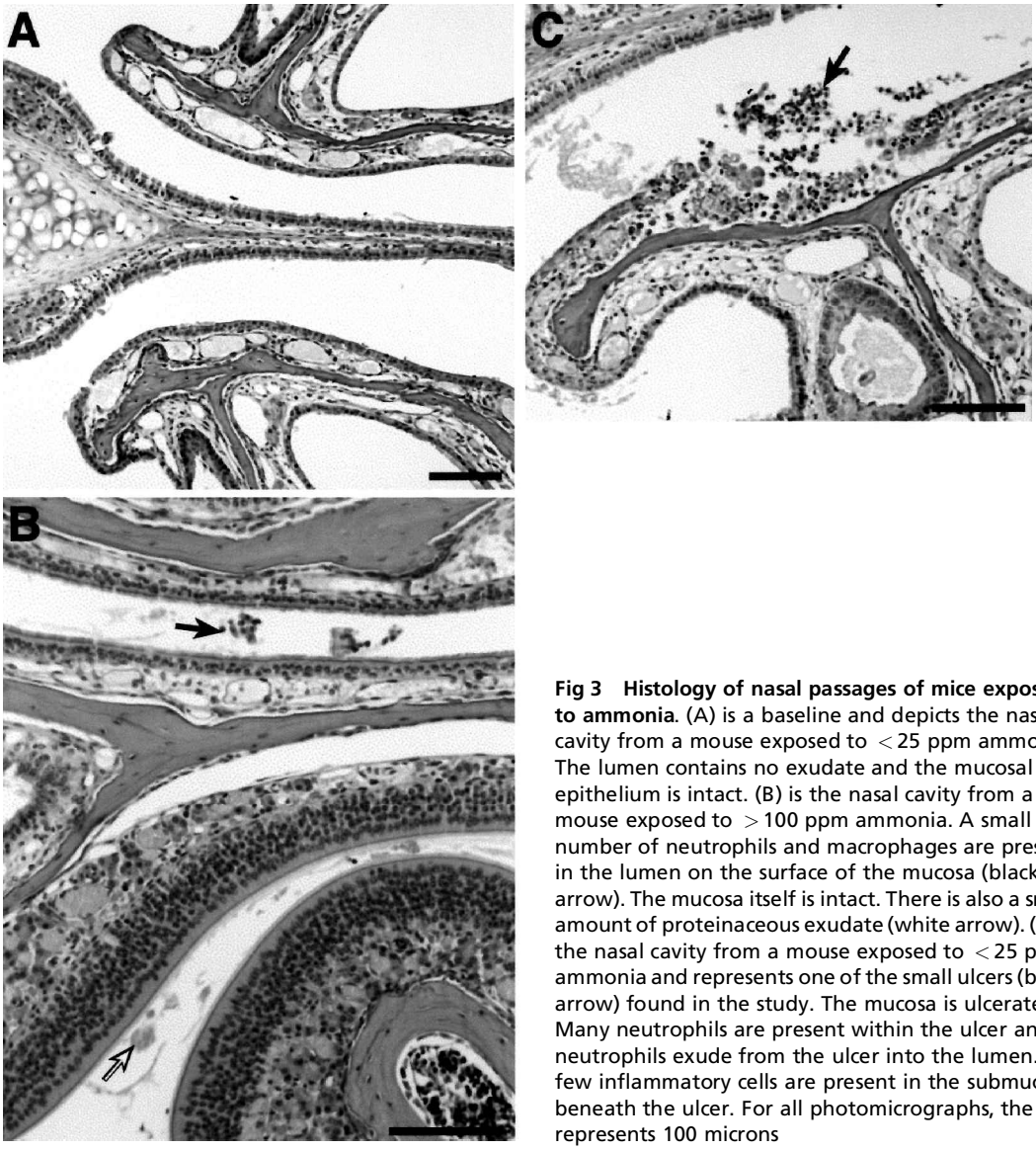

Fig 3 Histology of nasal passages of mice exposed to ammonia. (A) is a baseline and depicts the nasal cavity from a mouse exposed to <25 ppm ammonia. The lumen contains no exudate and the mucosal epithelium is intact. (B) is the nasal cavity from a mouse exposed to >100 ppm ammonia. A small number of neutrophils and macrophages are present in the lumen on the surface of the mucosa (black arrow). The mucosa itself is intact. There is also a small amount of proteinaceous exudate (white arrow). (C) is the nasal cavity from a mouse exposed to <25 ppm ammonia and represents one of the small ulcers (black arrow) found in the study. The mucosa is ulcerated. Many neutrophils are present within the ulcer and neutrophils exude from the ulcer into the lumen. A few inflammatory cells are present in the submucosa beneath the ulcer. For all photomicrographs, the bar represents 100 microns

affect the health of the animal. Indeed, pup mortality was higher when cages were changed every 7 days than when they were changed every 14 or 21 days. The ventilation rate of 60 ACH provided the optimum environment for mouse reproduction and growth under the conditions of these experiments.

Reduced frequency of cage change

We found that reducing the frequency at which cages were changed had no significant impact on the health of the animal. Reduced change frequencies of every 14 or 21 days did not adversely affect weanling weight, animal growth, plasma corticosterone concentrations, immune function, breeder mortality, and breeder productivity. That there was no significant difference between the number of mice born under experimental conditions versus the comparison group is consistent with the conclusion that the breeders were not stressed, as oestrus cycling, ovulation, and implantation are vulnerable to the depressive effects of stress (Moberg 1985). We found, in fact, that while the number of pups

Table 6 Incidence of lesions in nasal passages of adults and weanlings

Targeted ammonia exposure concentration, ppm	Mice	Actual ammonia concentration, ppm mean ± SEM	% of mice having inflammatory cells/total No. mice	No. of mice having an ulcer or abcess
< 25	Young	2 ± 1	30% (3/10)	0
25–50	Young	29 ± 3	8% (1/13)	0
50–75	Young	64 ± 4	31% (5/16)	1
75–100	Young	94 ± 4	6% (1/16)	0
> 100	Young	358 ± 28	10% (1/10)	0
< 25	Adult	2 ± 1	15% (3/20)	1
25–50	Adult	32 ± 2.5	23% (5/22)	0
50–75	Adult	60 ± 3	22% (4/18)	1
75–100	Adult	94 ± 2	19% (4/21)	0
> 100	Adult	241 ± 22	25% (5/20)	1

born was not affected, pup mortality was highest when cages were changed every 7 days; mortality for breeding pairs was 3.4 ± 0.7 in cages changed every 7 days, 1.3 ± 0.4 in cages changed every 14 days, and 1.6 ± 0.5 in cages changed every 21 days. The difference between 7 and 14 days was statistically significant; the same was true for breeding trios, but the difference between 7 and 14 days was not statistically significant for breeding trios. These results suggest that reduced changing frequency from once every 7 days to once every 14 or 21 days is beneficial to pup survival.

Because of increased pup survival, reducing the cage change frequency may benefit strains that have a high percentage of non-productive matings, a low born-to-wean ratio, or are difficult to handle. Reduced cage changing frequency may reduce the number of litters lost because of disturbances when they are newborns. On the other hand, a reduced cage change frequency may not be appropriate for diabetic mice or other strains that soil their cages excessively. Though the results of the health of the animal for a 21-day cage change frequency were virtually the same as those for a 14-day schedule, a 14-day

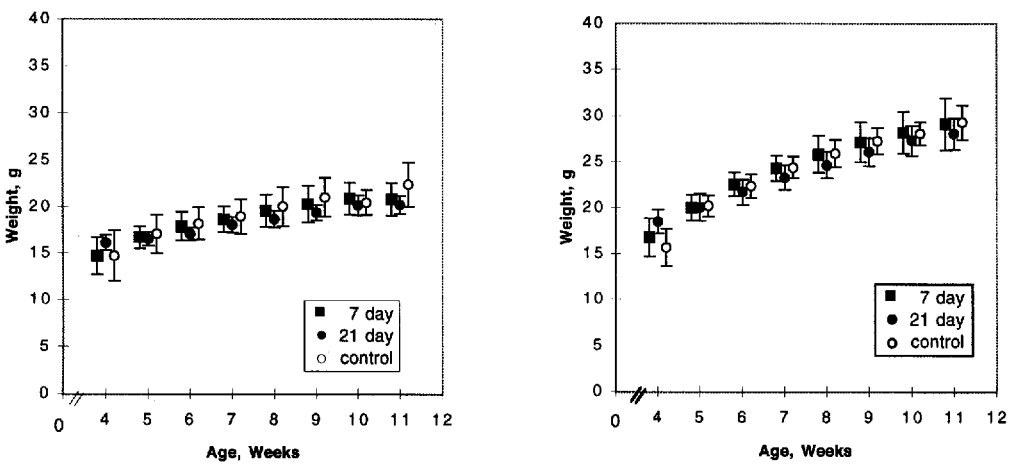

Fig 4 Growth curve of female (left) and male (right) offspring from age 4 weeks through age 11 weeks. Offspring were from pair-mated breeders housed at 60 air changes/h and changed once every 7 or 21 days. Weight data from offspring changed once every 14 days showed a similar growth pattern, and was omitted from the graph in the interest of clarity. Comparison mice were changed once every 7 days and housed in static cages

schedule may be more suitable to the logistical demands of routine animal care.

In addition to the health of the animal, other factors must be considered when reducing cage change frequency. The type of bedding, cage dumping and cleaning practices, and control of animal odours and allergens are also important. For example, changing the bedding of white pine (*Pinus strobus*) shavings only once every 21 days resulted in a hard-packed bedding in some areas of the cage, which may require extra cleaning. Physical properties of the bedding such as absorbency and particle size may also impact the optimal cage changing frequency. Additionally, because workers are exposed to allergens primarily when they change cages, they may be exposed to less allergen if they change cages only once every 14 or 21 days. Changing cages less frequently may result in greater allergen accumulation in the cages, but changing cages under ventilated hoods should reduce worker exposure to those allergens (Kacergis *et al*. 1996, Gordon *et al*. 1997, Reeb-Whitaker *et al*. 1999).

Cage ventilation rates

Huerkamp *et al*. (1994) reported no differences in fecundity or fertility between mice housed in unventilated and ventilated cages, both changed weekly. While we did not find differences in breeding fecundity between mice housed in ventilated cages compared with the unventilated group in this study, we did find that, in general, reproductive success was best when cages were ventilated at 60 ACH. In cages with breeding pairs, the number of pups weaned/dam was highest at 60 ACH (significantly higher than at 30 ACH); in cages with breeding trios, cage ventilation rates did not affect the number of pups weaned per dam. Pre-wean mortality was higher at 30 ACH than at 60 and 100 ACH (Table 2).

Ventilation rates of 100 ACH seemed to reduce the weights of weanlings from breeding pairs but not from breeding trios: weanlings born from breeding pairs weighed significantly less if housed at 100 ACH than if housed at 30 ACH and in the case of males at both 30 and 60 ACH. Perhaps the increased airflow at 100 ACH affected pup weights from only breeding pairs because there are fewer mice, and therefore less total body heat, in breeding pair cages. Consistent with this concept, air temperatures tended to decrease as the air change rate increased. However, conservation of body heat through huddling is more significant than air temperature.

Cage ventilation rates also affected the ammonia concentrations differently in cages with breeding pairs than in cages with breeding trios. When cages with breeding pairs were changed every 14 days, concentrations of ammonia could be kept at an average of less than 25 ppm if the cages were ventilated at 60 and 100 ACH. However, to maintain concentrations less than 25 ppm in cages with breeding trios that were changed every 14 days, cages needed to be ventilated at 100 ACH. Perhaps the high moisture and ammonia content in these cages required higher ventilation rates to keep ammonia concentrations down.

We were surprised to find that ammonia concentrations were higher in cages changed every 7 days and ventilated at 30 ACH compared with concentrations observed in static control cages (Table 1, Fig 2a). We have no satisfactory explanation for these results. Thirty ACH is the lowest setting possible in these cages and is not the recommended ventilation rate suggested by the manufacturer. Should it become economically essential to consider ACH below 60, more

Table 7 The effect of long-term reduced change upon measured immune function

	Mean % positive cells ± SEM	
Cell types	7-day change	14-day change
aCD8 pos.	6.84 ± 0.63	7.77 ± 0.48
aCD4 pos.	2.18 ± 0.12	2.49 ± 0.12
Γδ T cells	0.85 ± 0.07	0.80 ± 0.04
PMN	8.21 ± 0.82	9.65 ± 0.48
Monocytes	17.3 ± 1.67	16.1 ± 0.89
Mature B	59.0 ± 2.41	58.5 ± 1.25
Plasma cells	1.09 ± 0.42	0.61 ± 0.08

PMN = polymorphonucleated granulocytes; CD8 positive cells– cytotoxic T cells; CD4 positive cells–T helper cells. The number of mice in each group is four

work will be needed to determine whether lower rates are feasible.

Cage microenvironment

Overall, even at cage change frequencies of 14 and 21 days, we were able to keep humidity and temperature well within the ranges commonly recommended or accepted as optimum for the health of the animal. *The Guide for the Care and Use of Laboratory Animals* (National Research Council 1996) recommends that rodents be maintained at a relative humidity range of 30–70% and a temperature range of 18–26°C. Our values were within these ranges and concurred with the results of other studies that evaluated the effects of environmental factors on laboratory animals. Yamauchi *et al.* (1981) reported no difference in the number of rat litters born, litter sizes, weaning rates, or delivery rates when housed within the room temperature range of 14–28°C. However, when the rats are exposed to high temperatures of 30 and 32°C, litter size and weaning rates are low; rats exposed to temperatures below 18 or above 30°C grow slowly (Yamauchi *et al.* 1981). Donnelly (1989) reported that mice housed within a relative humidity range of 40–70% have no difference in litter sizes and weanling weight. However, at the low relative humidity concentration of 40%, survival to wean and growth rates are adversely affected (Donnelly 1989). Exposure guidelines for carbon dioxide do not exist, but the carbon dioxide concentrations recorded in this investigation were similar to or less than those published in other studies (Serrano 1971, Corning & Lipman 1991, Perkins & Lipman 1996, Reeb *et al.* 1997).

Effects of ammonia on the nasal passages

We found no pathological changes in the nasal passages of mice exposed to high concentrations of ammonia (Table 6). Thus, our results are at variance with somewhat similar studies in rats (Broderson *et al.* 1976) in which mild lesions consisting of epithelial hyperplasia accompanied by cell death and submucosal oedema, congestion, and fibrosis were reported in association with exposure to 150–250 ppm ammonia. The lesions involved a substantial portion of the nasal and olfactory mucosa. These differences may be due to: the exposure period being at least 35 days compared with about 10 days for our mice, that the rats were continuously exposed, or due to species differences. Similarly, Buckley *et al.* (1984) found mild lesions in mice exposed to 303 ppm of ammonia, but of a more inflammatory nature. Again, differences in the experimental design, such as the type of exposure and strain of mouse, could account for the differences.

There are no specific guidelines for exposure limits of mice to ammonia, probably because the literature is not clear on what levels are harmful. This is a major gap in our knowledge, one that we have attempted to study in order to make appropriate decisions about maintaining a healthy environment for mice. Experiments on the effects of ammonia occur with three different basic sets of conditions. First, rodents are either infected with respiratory pathogens or free from such infections, the effects of which have been reviewed recently by Lipman (1999). Second, ammonia exposure is either at a constant concentration or at the fluctuating concentration that occurs naturally. Third, rodents are either in contact with bedding and excreta or suspended above excreta in mesh bottom cages. Each of these different conditions strongly influence the outcome of ammonia exposure, with the length of exposure being an additional significant variable.

The threshold limit value (TLV) for humans, which is 25 ppm (American Conference of Governmental Industrial Hygienists 2000), is often cited as the desirable benchmark limit for environmental ammonia exposure to mice. However, species differ widely in their ability to tolerate ammonia. Guano bats maintain colonies in caves with ammonia levels ranging from 100–1800 ppm and survive experimental exposure of 5000 ppm for 3 days. In contrast, humans cannot safely tolerate concentrations of 100 ppm for more than an hour (Studier *et al.* 1967). Mice fall into an intermediate range, surviving one hour at 3440 ppm (Kapeghian *et al.* 1982) and 16 h at 1000 ppm (Studier *et al.* 1967). Thus, the application of human threshold limit values

to other species does not accurately reflect their tolerance.

Strains of mice also differ in their response to ammonia (Schaper 1993). For example, the level of ammonia that results in a 50% reduction of the respiratory rate (RD_{50}) is 303 ppm in Swiss Webster mice (Barrow *et al.* 1978) and 790 ppm in BALB/c (Tomas *et al.* 1985). The RD_{50} observed in rodents for many chemical irritants was compared with the exposure level at which humans experience intolerable eye, nose, and throat irritation (Barrow *et al.* 1978). For many chemicals, human exposure of 0.1 to 0.01 of the rodent RD_{50} correlates with the TLV for humans (Schaper 1993). Using this guideline, we would predict that 10 times the TLV of 25 ppm for humans would irritate mice. However, mice demonstrated no aversion to 500 ppm ammonia (Wood 1979) nor showed signs of irritation at 500 ppm (Studier *et al.* 1967) or 700 ppm (Wood 1979). Exposure of mice to RD_{50} of 303 ppm ammonia continuously for 6 h/day for 5 consecutive days resulted in mild degenerative and moderate inflammatory changes in the nasal mucosa, consisting of scattered infiltration of neutrophils in the epidermis and lamina propria of the nasal, but not the olfactory epithelium (Buckley *et al.* 1984). We did not find any pathological changes in the nasal passages of mice exposed to ammonia levels approaching 400 ppm, but our study differed from that of Buckley and co-workers because our mice were exposed to 14-day cycles of increasing ammonia which culminated on the day of weaning. A mouse can reduce its exposure to ammonia because there is an ammonia gradient in the cage from the nest to the food hopper (Eveleigh 1991) as well as vertically (Broderson *et al.* 1976). Although we observed no tendency of the mice to seek cage locations with reduced ammonia, the mice do move about the cage and climb onto the cage lid and hence may reduce their exposure.

Further complicating the interpretation of the significance of exposure to ammonia is the phenomena of adaptation, which is known to occur in both humans (American Conference of Governmental Industrial Hygienists 1991) and animals (Barrow *et al.* 1986). Adaptation or tolerance to respiratory irritants does occur in rodents. For example, nasal irritation and severe inflammatory changes occurred in the upper respiratory tract of 'specific pathogen free' rats exposed to 500 ppm of ammonia at 3 weeks of exposure, but not at 8 weeks (Richard *et al.* 1978).

While there is reason to be concerned that low ammonia levels could affect certain research findings, we conclude that the ammonia levels experienced by the mice in our breeding trials did not have a negative impact on growth rate, breeding efficiency or the health of the animal, nor did the deliberately higher level of exposure of the ammonia trials result in demonstrable pathological changes. Clearly more research on the effects of ammonia on both pathogen-free and infected rodents is necessary to better understand what ammonia concentrations may be harmful to mice.

The technology utilized for ventilating the animal cage differs among the various cage manufacturers. The method of air supply to and exhaust from the cage as well as the rack may influence airflow velocities and patterns within the cage. For example, in the system used in this study, air enters and leaves the cage through a filter cover on the top of the cage. Because the cover diffuses the air entering the cage, intra-cage air velocity is lower than in systems where air is injected directly into the cage (Tu *et al.* 1997). Because of the differences in cage system design, the microenvironment concentrations and animal health status, the findings reported here may differ from those observed in other laboratory animal housing ecosystems. Factors such as bedding material, watering systems, and animal phenotype may also influence the cage microenvironment and the health of the animal.

In conclusion, mechanically-ventilated cages offer the opportunity to control the cage microenvironment for the benefit of both mice and animal facility workers. They also call for re-examination of time-honoured animal husbandry practices, such as those concerning the frequencies at which cages are changed. Reducing cage change frequency from once every 7 days to once every 14 or 21 days had no adverse health effects on

C57BL/6J mice housed in ventilated cages. Indeed, changing these cages every 7 days, the same schedule at which static, non-ventilated cages are changed, resulted in higher pup mortality than when they were changed every 14 or 21 days. Furthermore, a ventilation rate of 60 ACH in these cages provided mice with the optimal cage microenvironment. Our results suggest that reducing the frequency of cage changing in ventilated cages is a viable husbandry practice and should be further explored.

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