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The effect of cage ventilation rate on the health of mice housed in Individually Ventilated Cages

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Summary

The number of air changes per hour (ACH), an important index for individually ventilated cages (IVC), strongly affects the cage microenvironment and the health of laboratory animals. The objective of this study was to determine whether high or low cage ventilation adversely affects the health of mice housed in IVC systems and to identify cage ventilation rates suitable for the welfare of mice. We tested three different cage ventilation rates (40, 60, and 80 ACH) for 3 weeks in an IVC system. The temperature, relative humidity and ammonia concentrations in the cages were measured daily. The indices used to assess mouse health at specific time points throughout the study were body weight, stress hormones, T lymphocyte subsets (CD4 and CD8), immunoglobulins (IgG, IgM and IgA) and immune cells. There were no significant differences in body weight, growth hormones, immunoglobulin and T lymphocyte subsets in the IVC groups compared with the control group. The concentrations of corticosterone and epinephrine on day 7 of cage ventilation at 80 ACH were significantly higher than those in the control group ($P < 0.05$). Mice housed in 80 ACH cages had the lowest immune cell counts among all groups, and the numbers of lymphocytes and neutrophils were significantly lower than those in the control group ($P < 0.05$). In summary, cage ventilation at 60 ACH provided an optimum cage microenvironment for mouse health and welfare.

Introduction

Technological developments in recent decades have yielded a novel housing system for laboratory animals, called 'Individually Ventilated Cages' (IVCs). At present, IVCs are commonly and increasingly used. IVC systems have numerous benefits, including relatively low investment costs, easy operation and high degree of containment, which can protect animals against infections, substantially reduce exposure to laboratory animal allergens and improve the health of staff (Gordon et al. 2001; Myers et al. 2003; Schweitzer et al. 2003; Compton et al. 2004). In particular, through effective cage ventilation, IVC systems allow for a relatively healthy microenvironment for laboratory animals.

The number of air changes per hour (ACH), an important technical index of IVC systems, substantially affects the cage microenvironment and the health of laboratory animals. Compared with static (unventilated) microisolator cages, IVC cages have lower ammonia, lower CO_2, lower humidity and higher O_2 (Memarzadeh et al. 2004; Rosenbaum et al. 2010; Nagamine et al. 2012). Cage air exchange can

reduce the accumulation of noxious gases, ensure the drying of bedding and provide a more healthy and comfortable cage microenvironment. High concentrations of ammonia and CO_2 in cages can induce mouse stress responses and physiological and hormonal changes, such as increased respiration and elevated corticosterone, thus severely threatening animal health (Krohn and Hansen 2000; Krohn et al. 2003).

A previous study has evaluated the effects on the cage microenvironment of varying the ventilation rates from 30 to 100 ACH; the authors concluded that 30 ACH is sufficient to maintain an adequate microenvironment when bedding is changed weekly (Reeb et al. 1998). The authors performed further research to determine whether the cage changing interval could be prolonged without adversely affecting mouse health which was assessed on the basis of breeding performance, weanling weight and growth, plasma corticosterone levels, immune function and histological examination (Reeb-Whitaker et al. 2001). Cage changes once every 14 days and a ventilation rate of 60 ACH were found to provide the optimum conditions for animal health and practical husbandry.

In addition, one study has shown that mice reject cages with a high number of air changes (100 ACH), possibly because the high ventilation rate induces a high air speed that may cause stress and discomfort (Baumans et al. 2002). Another investigation of the effects of air changes of 50, 80, and 120 ACH in rats has reported that the number of air changes in each cage should be kept below 80 ACH to avoid affecting rat physiological indexes (Krohn et al. 2003; Krohn and Hansen 2010). Therefore, the cage ventilation rate is an important factor affecting the physiology and behavior of laboratory animals, and the validity and reproducibility of scientific data.

Although several studies have assessed cage microenvironmental parameters under variable cage ventilation rates (Baumans et al. 2002; Reeb et al. 1998; Reeb-Whitaker et al. 2001), few studies have simultaneously and systematically examined the effects of cage ventilation rate on the cage microenvironment, animal health status and welfare. In this investigation, we sought to determine whether high or low cage ventilation adversely affects the health of mice housed in IVC systems and to identify cage ventilation rates suitable for mouse health and welfare. In addition, we sought to understand better the environment of laboratory mice and to provide a scientific basis for the development of updated guidelines for animal health and practical husbandry.

Materials & Methods

Animals and housing conditions

Female ICR mice (n=100; age, 6 weeks; weight, 24-28 g) were obtained from a commercial vendor (ChangshengBio Corp, Benxi, Liaoning), and randomly grouped and housed in polyphenylsulfone (28.5 cm × 15 cm × 13.5 cm) cages within an IVC rack. According to the results of health surveillance programs performed by the vendor and our research institution, the mice were free of 15 viruses (mouse hepatitis virus, mouse parvoviruses, reovirus, theiler's mouse encephalomyelitis virus, ectromelia virus, mouse rotavirus, thymic virus, pneumonia virus of mice, sendai virus, murine cytomegalovirus, murine norovirus, lymphocytic choriomeningitis virus, lactic dehydrogenase-elevating virus, hantavirus, and mouse adenovirus), 17 bacterial species (including Helicobacter spp), two Mycoplasma spp, mouse ectoparasites and endoparasites, and encephalitozoon cuniculi. Female mice were chosen because they have a lower potential for intra-cage aggression than males; body injury due to aggression can affect hormone levels and immune function.

The IVC system (HongtengBio Corp, Dongguan, Guangdong) was placed in a laboratory animal barrier system, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The animal room directly collected outdoor air through high efficiency particulate arresting (HEPA) filtration and was maintained at 15 ACH, 23 - 27 °C and 30 - 70% relative humidity. The light cycle was 12 h light and 12 h dark. The cage air pressure was negative with respect to the room. During the study, all cages were equipped with approximately 150 g of autoclaved wood shaving bedding (Keao Corp, Beijing, China), water bottles and Co60 sterilized feed (Keao Corp, Beijing, China). The cages and bedding were changed every 7 d.

Experimental design

We studied three different cage ventilation rates (40, 60, and 80 ACH) in three IVC systems of the same model. Each cage contained five mice, and six cages were located within each rack. The experiment lasted 3 weeks. The environmental parameters analyzed in this study consisted of daily ammonia levels, and the temperature and relative humidity in the cages and the room. Indices used to assess the health of mice at specific time points throughout the study were body weight, stress hormones (corticosterone, growth hormone, and epinephrine), T lymphocyte subsets (CD4 and CD8), immunoglobulins (IgG, IgM and IgA)

and immune cells. In addition, two open-top cages in the animal room were used as a control. At the conclusion of the experiments, animals were euthanized with carbon dioxide gas. All experimental procedures were approved by IACUC of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Cage microenvironment

The temperature and relative humidity in the cages were measured daily with a combined temperature and humidity detection instrument (TES-1360A Humidity/Temperature Meter, TES, Taibei, Taiwan). The measurement accuracy of this device can range from ±3 %RH and ±0.8 °C. For sampling, the device was quickly placed into cages after the lid was opened and air was sampled for a minimum of 5 min, and the maximum value was reported. The room was always measured first by sampling at a height of 1 m in the center of the room for 5 minutes. Cage and room ammonia levels were monitored daily with a pumping ammonia concentration detector (CH100-NH3, Chuchuang, Jinan, Shandong). The measurement accuracy of this device ranged from ±2 ppm, and the maximum detectable concentration was 200 ppm. Ammonia concentrations were measured using the same method as that for temperature and humidity. After data were obtained, the cages were returned to the ventilated rack. Both devices had been calibrated by professional inspection institutions and were within the valid verification period.

Body weight

Mice were weighed at the same time on days 0, 7, 14, and 21. The same electronic scale (PL203, Mettler toledd, Shanghai, China) was used to weigh all mice over the course of the study.

Hormone levels

To assess the response of the sympathetic nervous system to different cage ventilation rates, we measured three stress-related hormones in the mice: epinephrine, corticosterone and growth hormone. Blood was sampled between 08:30 and 10:00 h before cage change on the last day of each week. According to a method described by Golde et al. (2005), 0.5ml blood samples were obtained by an experienced technician from the submandibular vein without anesthesia, to reduce animal stress and avoid fluctuations in hormones during sample collection. Serum samples were measured according to the protocol provided by the manufacturer of the enzyme linked immunosorbent assay kits (JianchengBio, Nanjing, Jiangsu). The kits contained internal controls, and a standard curve was calculated to determine sample values.

Immune function

To assess the long-term effects of cage ventilation rates on immune function in mice, 0.2 mL blood samples were placed into EDTA anticoagulation tubes on the last day of the experiment. An automated blood physiological analysis system (BC-2800Vet, Mindray, Shenzhen, Guangdong) was used to determine the number of monocytes, leukocytes, neutrophils and lymphocytes. This test was performed immediately after blood collection to ensure data accuracy. Another aliquot of the blood samples was used to determine the concentration of immunoglobulins and the T lymphocyte subset. Immunoglobulin is an important component of the immune system, and its concentration reflects immune function to some extent. Serum samples were measured according to the protocol provided by the manufacturer of the enzyme linked immunosorbent assay kits (LiankeBio, Hangzhou, Zhejiang). The kits contained internal controls, and a standard curve was calculated to determine sample values.

Statistical analysis

All statistical analysis was performed with IBM SPSS version 19.0 for Windows, and values are presented as the mean ± SD. Statistical significance was defined as a P value of less than 0.05. Temperature and humidity data were analyzed by using a general

Table 1. Measured variables, frequency of sampling and sampling methodologies

Measured variable	Frequency	Methodology
Ammonia	Daily	Pumping ammonia concentration detector
Temperature and humidity	Daily	TES-1360A Humidity/Temperature Meter
Body weight	Days 0, 7, 14, 21	0.1-g electronic scale
Stress hormones	Days 7, 14, 21	ELISA
T lymphocyte subset	Day 21	ELISA
Immunoglobulin	Day 21	ELISA
Immune cell	Day 21	Automated blood physiological analysis system

linear mixed model for repeated measures, with day as a within-cage factor and cage ventilation rate as a between-cage factor. After goodness-of-fit indices for several covariance models were compared, a first-order autoregressive model was chosen to measure the within-cage covariance over time. Ammonia levels, body weight, hormone levels, T lymphocyte subset, immunoglobulins and immune cells were analyzed to determine the statistical significance of the data according to the cage ventilation rate group by using one-way ANOVA. A complete list of variables measured, frequency of sampling and methodology used to obtain the data is given in Table1.

Results

Temperature

The temperature within cages was significantly associated with the number of days and the cage ventilation rate ($P < 0.001$). There were significant differences in temperature among the three cage ventilation groups ($P < 0.05$). Across all days, the cage temperature for all three ventilated groups was consistently significantly higher than room temperature ($P < 0.05$), and the cage temperature decreased with increasing cage ventilation (Figure 1).

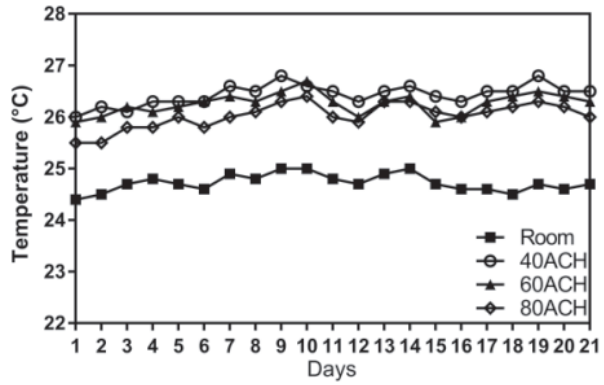

Figure 1. Temperature (°C) of each ACH group and room, daily during 21 days. Each line represents the average value of the 6 cages in each ACH group.

Humidity

The relative humidity within cages was significantly associated with the number of days and the cage ventilation rate ($P < 0.001$). There were significant differences in relative humidity among the three cage ventilation groups ($P < 0.05$). Across all days, the relative humidity within cages for all three groups was consistently significantly higher than the room levels ($P < 0.05$), and the cage relative humidity decreased with increasing cage ventilation (Figure 2).

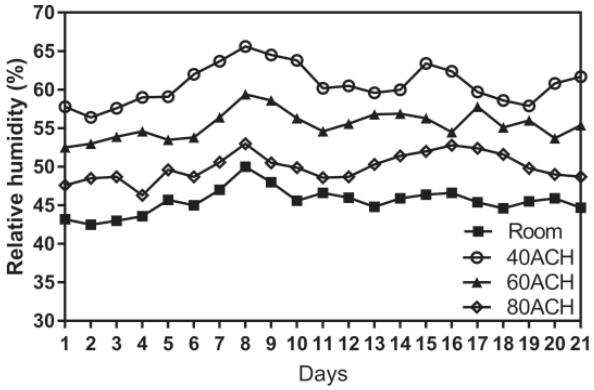

Figure 2. Relative humidity (%) of each ACH group and room, daily during 21 days. Each line represents the average value of the 6 cages in each ACH group.

Ammonia

The room ammonia levels were 0 ppm for all sampling days. The cages showed a detectable increase in ammonia on day 3, and the ammonia concentrations steadily increased thereafter until the time of the cage change on day 7 (Figure 3). The highest average level of ammonia within cages was recorded on day 7 in the 40 ACH group (approximately 22 ppm), and the ammonia levels within cages decreased with increasing cage ventilation rates. The average ammonia levels on day 6 and 7 showed significant differences among the three cage ventilation groups ($P < 0.05$), and the average ammonia level on day 5 in the 40 ACH group was significantly higher than that in the 60 and 80 ACH groups ($P < 0.05$).

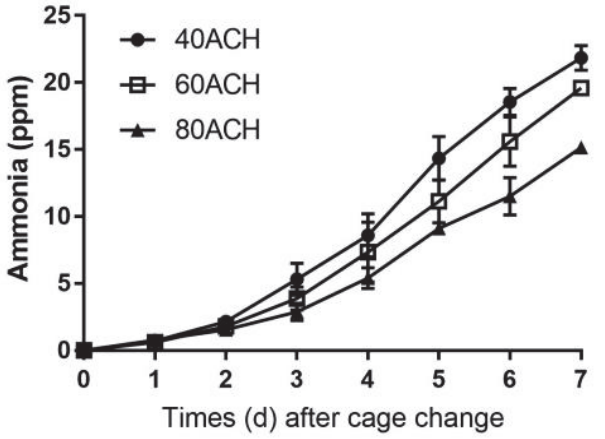

Figure 3. Ammonia concentrations for various ventilation rate groups after cage change. Each line represents the average value for 3 weeks measured for 6 cages per group.

Body weight

Weight loss due to chronic stress was not observed in any mice. Average body weight gains from day

Table 2. The effect of cage ventilation rate on measured immune function

		Cage ventilation rate		
Immune indices	**Control**	**40 ACH**	**60 ACH**	**80 ACH**
IgA (ng/ml)	64.81 ± 4.38	65.07 ± 6.97	67.80 ± 5.65	64.27 ± 8.95
IgG (ng/ml)	553.42 ± 79.58	550.59 ± 78.61	527.23 ± 67.81	485.39 ± 51.69
IgM (ng/ml)	3.91 ± 1.03	4.28 ± 0.79	3.43 ± 1.64	3.25 ± 1.40
CD4 (U/ml)	12.68 ± 1.15	13.46 ± 1.07	13.12 ± 1.14	13.58 ± 1.40
CD8 (U/ml)	112.41 ± 8.98	114.24 ± 4.67	111.22 ± 10.47	114.35 ± 11.90

CD4 positive cells, T helper cells; CD8 positive cells, cytotoxic T cells.

Table 3. The effect of cage ventilation rate on measured immune cells

		Cage ventilation rate		
Cell types($\times 10^9$/L)	**Control**	**40 ACH**	**60 ACH**	**80 ACH**
Leukocytes	5.76 ± 0.99	4.82 ± 0.84	5.47 ± 0.80	4.45 ± 1.15
Lymphocytes	4.08 ± 0.74	4.58 ± 1.23	4.56 ± 0.86	2.62 ± 0.62*
Monocytes	0.27 ± 0.14	0.20 ± 0.13	0.22 ± 0.04	0.18 ± 0.08
Neutrophils	1.35 ± 0.39	1.18 ± 0.45	1.62 ± 0.26	0.83 ± 0.35*

*Value is significantly different from control ($P < 0.05$).

0 to day 21 were as follows: control 8.38±1.15 g, 40 ACH 8.06±0.89 g, 60 ACH 8.90±1.85 g, and 80 ACH 7.88±1.33 g. No significant differences in weight gain were found across the groups, and cage ventilation did not significantly affect weight gain. The weight gain at 80 ACH was lower than that at other cage ventilation levels, although the result was not statistically significant.

Hormone levels

The corticosterone levels under cage ventilation at 40 ACH and 60 ACH were not significantly different from those of the control group at any time point, whereas the level on day 7 under 80 ACH was significantly higher than that of the control group ($P < 0.05$). No statistically significant trends were detected in corticosterone concentration over time in each ACH group (Figure 4).

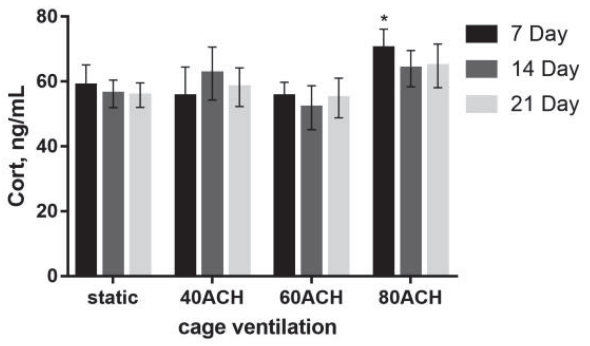

Figure 4. Corticosterone levels at 7, 14 and 21 days in each ACH group. An asterisk above a column denotes a statistically significant difference compared with the control ($P < 0.05$).

Significant differences were not detected for growth hormone at any time point, and no statistically significant trends were detected in growth hormone concentration over time in each ACH group (Figure 5).

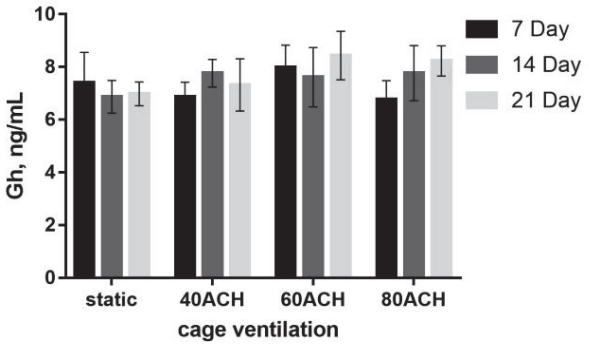

Figure 5. Growth hormone levels at 7, 14 and 21 days in each ACH group.

The epinephrine levels under cage ventilation at 40 ACH and 60 ACH were not significantly different from those of the control group at any time point, whereas the levels on days 7 and 14 under 80 ACH were significantly higher than those of the control group ($P < 0.05$). No statistically significant trends were detected in epinephrine concentration over time in each ACH group, but the epinephrine concentration at 80 ACH tended to decrease over time (Figure 6).

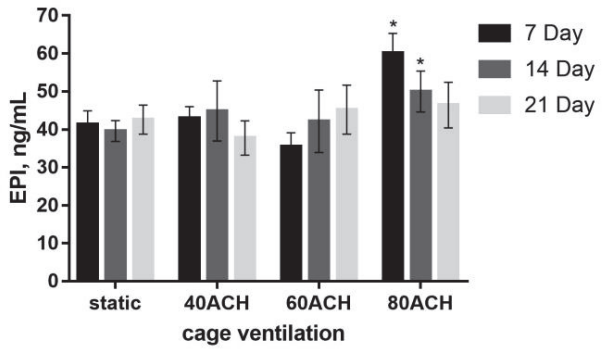

Figure 6. Epinephrine levels at 7, 14 and 21 days in each ACH group. An asterisk above a column denotes a statistically significant difference compared with the control (P < 0 .05).

Immune function

The results from the enzyme linked immunosorbent assays showed no significant differences in immunoglobulins and T lymphocyte subset among the ACH groups (Table 2). As shown in Table 3, mice housed in 80 ACH cages had the lowest immune cell counts, and the numbers of lymphocytes and neutrophils were significantly lower than those in the control group (P < 0.05). No significant differences were detected in the other ACH groups.

Discussion

Housing systems can have significant effects on laboratory animal physiology and behavior, two aspects closely related to mouse welfare. IVC systems can maintain higher air quality in the cage environment through effective ventilation rate. However, different cage ventilation rates have varying effects on the environment within cages. In this study, a systematic simultaneous examination of cage microenvironment, health status and welfare relative to the cage ventilation rate was performed to determine whether high or low air exchange might compromise the well-being of mice and to identify cage ventilation rates suitable for mouse welfare.

The temperatures measured in this study remained within the boundaries suggested in the guide for European laboratory-housed mice. In agreement with results from previous studies (Spangenberg et al. 2014), the ventilated cage temperature remained higher than the room temperature, perhaps because the lids of the IVC were closed. There were significant differences in temperature among the three cage ventilation groups. Although the temperature within cages differed only slightly between the test groups, the effects of temperature on mouse health cannot be ignored. A study has shown that as ambient temperature decreases, the mean blood pressure, heart rate and pulse pressure significantly increase in mice and rats, and mice have greater sensitivity to these temperatures changes (Swoap et al. 2004).

According to current guidelines, the relative humidity of the microenvironment for rodents should remain within 30-70%; levels above or below this range may increase preweaning mortality in mice (Clough 1982). Throughout this study, the relative humidity in the room and in cages remained within this recommended range. There were significant differences in relative humidity among the cages in the three ventilation groups, and cages at 80 ACH had the lowest humidity levels. This result suggests that a higher cage ventilation rate removes excess moisture from cages. Furthermore, the room ventilation rate also affects the humidity in cages, which has been found to significantly decrease from 55% relative humidity at 5 ACH to 36% relative humidity at 20 ACH (Reeb et al 1997). Another study has compared the cage microenvironment under room ventilation rates of 5 to 6 ACH versus 10 to 12 ACH and has concluded that higher room ventilation rates provide a comfortable housing environment for mice (Geertsema et al. 2015). The most important reason for controlling relative humidity inside the cage is that excessive moisture enhances the proliferation of urease-positive bacteria and increases ammonia production (Memarzadeh 2005). A relative humidity substantially significantly exceeding 35% dramatically increases ammonia generation rates in static mouse caging (Freymann et al. 2015). A study evaluating two levels of relative humidity (35% and 75%) within mouse cages has found that lower humidity results in lower generation of ammonia (Perkins and Lipman 1996).

At room temperature, ammonia is a colorless gas with a distinctive pungent odor, and it is a severe irritant to the respiratory tract, skin and mucous membranes (Perkins and Lipman 1995). Ammonia is produced by the conversion of urea by urease, which may be present in bedding or produced by fecal bacteria (Freymann et al. 2015). Studies have demonstrated that prolonged exposure to high ammonia concentrations can cause histologic changes in the respiratory tract and promote the growth of pathogenic bacteria; in addition, rats exhibit better reproductive performance and a lower incidence of pneumonia at lower ammonia levels (Höglund and Renström 2001; Teixeira et al. 2006).

In our study, the cages showed a detectable increase in ammonia on day 3, and the ammonia concentrations steadily increased thereafter until the cage change on day 7. After the cage change, ammonia returned to very low levels. These findings of cyclical fluctuations are consistent with results from previous studies (Silverman et al. 2009; Mexas et al. 2015; Eichner et al. 2017). The 40 ACH cages had the highest levels of ammonia, which may cause discomfort and stress in mice, and adversely affect mouse health. The 80 ACH cages had the lowest ammonia levels, thus suggesting that higher air exchange rates effectively decrease ammonia concentration in cages.

In addition to the factors discussed in this study, the cage change frequency, the amount and type of bedding and animal density can affect ammonia levels in cages (Gordon 2004; Smith et al. 2004). Prolonging the cage change frequency may lead to bacterial proliferation and increased ammonia concentrations (Allison et al. 2011). One study has evaluated the effects of three bedding volumes (low, medium and high) on cage microenvironment and mouse health, and has concluded that low bedding volume is associated with higher ammonia and humidity levels (Rosenbaum et al. 2009). High housing densities may adversely affect animal health, for example, by compromising air quality inside the cage (Divincenti et al. 2012).

Although exposure to ammonia may influence rodent health, precise exposure data and tolerable ranges are unknown (Silverman et al. 2008). There are no specific guidelines for the maximum ammonia concentration to which mice can be exposed, probably because the ammonia levels that are harmful are unclear, and human exposure standards may not be applicable to mice. In addition, research has shown that mice can cope with high ammonia concentrations within cages, and different strains of mice differ widely in their tolerance of relatively high ammonia levels (Gordon 2004; Green et al. 2008). Therefore, more research on the effects of ammonia on mice is necessary to better understand what ammonia concentrations may be harmful to mice.

Body weight is an important metric commonly used in animal experiments, as a nonspecific indicator of mouse health. Decreases in body weight may indicate that mice are stressed or that their health is impaired by a detrimental housing environment. In the current study, significant differences in body weight were not detected across the different ACH groups; weight gain at 80 ACH was lower than that at other cage ventilation rates, although the result was not statistically significant. This result may suggest that excessive cage ventilation negatively affects weight gain in mice.

Poor housing systems can cause stress to laboratory animals, affect physiological systems, and perturb biochemical stability. When animals are stressed, the hypothalamic–pituitary–adrenocortical axis and the sympatho-adrenomedullary system, which both have a key role in hormonal reactions to stress, are activated (Broom 1986). Adverse situations trigger responses of the adrenals, which result in an increase in glucocorticoid and/or catecholamine secretion (Moberg 2000). These increases are the front-line endocrine mechanisms to defend the organism against stressful situations (Mostl and Palme 2002). Corticosterone levels have been widely used as a physiological parameter reflecting animal health and welfare, and they represent the degree of damage to the body caused by chronic stress to some extent (Mostl and Palme 2002; Godfrey & Silverman 2009). In the current study, epinephrine levels on days 7 and 14 under 80 ACH were significantly higher than those of the control group, and the corticosterone level on day 7 under 80 ACH was significantly higher than that of the control group. The reason for these observations might be that the IVC with cage ventilation at 80 ACH produced more rapid air speeds in cages, which caused a stress response and adversely affected mouse health. Growth hormone is also associated with the stress response, one study has shown that acute stress decreases the secretion of growth hormone in the peripheral blood of adult rats; this effect is caused by the secretion of CRF (corticotropin-releasing hormone) from the hypothalamus, thus increasing somatostatin secretion (Eck and Kuhn 1992). In our study, growth hormone concentration was not significantly decreased at any time in each group.

In summary, our investigation demonstrated that cage ventilation at 60 ACH provided an optimum cage microenvironment for mouse health and welfare. The setting at 40 ACH was the lowest possible in the IVC system used in this experiment and is not the recommended because it cannot maintain a healthy cage microenvironment. A setting at 60 ACH may allow for a balance between maintaining high quality air in cages and not disturbing mouse health. When 80 ACH was set in the IVC system, the high air speed caused discomfort in the mice and affected their physiology. Because of the differences in cage system design, microenvironment conditions and animal health status, the results reported here may differ from those for other IVC systems. Therefore, further studies are necessary to better understand

the relationships among cage ventilation rate, cage microenvironment and mouse health.

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