Journal of the American Association for Laboratory Animal Science
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Vol 61, No 5
September 2022
Pages 432–440

Carbon Dioxide, Oxygen, and Ammonia Levels in Mouse and Rat Disposable IVC Removed from Mechanical Ventilation

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Maintenance of an appropriate microenvironment for rodents used in research is of paramount importance because changes in environmental parameters such as O_2 and humidity can influence animal health and welfare and potentially alter research results. Here we evaluated the microenvironment of mouse and rat disposable cages after removal from mechanical ventilation in order to guide recommendations for their use. Cages with sealed IVC lids, unsealed lids (partially ajar), and lids without the exhaust filter (for rats) or static lids (for mice) were removed from the ventilated rack and were thereafter monitored CO_2, O_2, and NH_3 levels. For mice, effects were investigated under both standard (set point of 72°F/22°C) and thermoneutral (set point of 82°F/28°C) temperatures. When IVC with sealed lids and group-housed C57BL/6J male mice were removed from ventilation under standard temperatures, CO_2 started at 6,600 ± 265 ppm at 0 h and rose to 42,500 ± 7,263 ppm at 1 h, with mice showing a visibly elevated respiratory rate in 1 of the 3 cages; CO_2 stabilized at 26,150 ± 3,323 ppm at 8 h. In contrast, CO_2 levels in cages with single mice were stable after 1 h (1,350 ± 409 ppm at 0 h, 9,367 ± 802 ppm at 1 h, and 8,333 ± 1,115 ppm at 8 h). Findings were similar at thermoneutral temperatures: sealed group-housed mice cages started at 3,617 ± 475 ppm at 0 h and rose to 39,333 ± at 5,058 ppm at 1 h, whereas sealed cages with 1 mouse started at 1,117 ± 247 ppm at 0 h and were 7,500 ± 1,997 ppm at 8 h. IVC with sealed lids and pair-housed Crl:CD(SD) female rats rose to 48,000 ± 2,828 ppm CO_2 and over 70% humidity within 1 h. By 3 h, IVC with sealed lids and singly housed rats had 40,167 ± 5,132 ppm CO_2 , and rats were displaying a visually elevated respiratory rate. O_2 levels had an inverse relationship with CO_2 levels. Removing the rat lid exhaust filter was not helpful. However, leaving the IVC lid ajar ameliorated the rise in CO_2 and fall in O_2 for both species. Therefore, IVC with sealed lids and group-housed mice should not be removed from ventilation more than 1 to 2 h; IVC containing pair- or singly-housed rats IVC should not be removed for more than 1 or 3 h, respectively. Whenever possible, such cages should be fitted with static lids, left partially ajar and monitored, or replaced on ventilation.

DOI: 10.30802/AALAS-JAALAS-22-000028

Introduction

Use of IVC systems for rodents used in research has helped to create standardized, secure, and stable microenvironments.[5] The purposes of ventilation are to provide optimal air quality and oxygen supply; to reduce the animal-related heat load particulates and waste gases in the cage, and to dilute allergens and other particulates.[5] Removing the cages from ventilation for experimental use greatly reduces air exchange. Commercially available IVC vary in the type of seal used, intake and exhaust area, inclusion of gaskets or latches, and other features that secure the cage and maintain the microenvironment. In our current cage system, the plastic lid is tightly fitted to cage interface and restricts air flow when the cage is removed from mechanical ventilation. This feature is characteristic of many disposable IVC, and its purpose is to protect the cage occupants and limit the potential for animal escape and biosecurity breaches. The microenvironmental conditions (temperature, humidity, CO_2, NH_3) of disposable ventilated cages on this type of mouse IVC rack have been described.[7,15,16] A selection of cage lids with different filter sizes and types is available for mice and rats, although only one option is available for rats in tall cages. Anecdotally, we noted that leaving rats in a sealed cage with IVC lid off ventilation for more than 1 to 2 h resulted in visible condensation on the cage wall, leading us to question how long rodents can appropriately be housed on a benchtop in these cage and lid configurations. Group-housed mice, when left for longer periods, also generated condensation on the cage wall, although static lids can circumvent condensation when longer durations or static caging is needed. In a previous study, housing mice in a similar cage system that had a lid with a smaller surface area led to the recommendations to limit cage removal from ventilation to 6 h or less, due to the development of hypoxic and hypercarbic conditions, and to instead use static lids.[13] Off-rack environmental conditions at the cage level depend on the cage type and lid seal, surface area of the filter to permit ventilation, biomass, behavior and activity of the animals, bedding type, diet, and macroenvironmental conditions.[10,12] In addition, tolerance to microenvironmental levels may vary among strains and individual animals. For example, differences in aversion to rising CO_2 levels in rats may be related to behavioral responses over time and situational experience.[1] However, information on microenvironmental parameters for the wider exhaust-filter IVC lid for mice used at our facility and types of interventions that may aid air exchange remain sparse, and no information is available for this type of rat caging. This information is important for developing interventional criteria and emergency actions during events such as power or rack failures.

Submitted: 28 Mar 2022. Revision requested: 26 May 2022. Accepted: 05 Jul 2022.
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The objective of the current study was to evaluate microenvironmental changes in CO_2, O_2 and NH_3 in disposable mouse and rat IVC when they are placed on the benchtop and to provide guidance regarding mitigation practices for singly and group-housed mice and rats. Evaluation of the microenvironment will inform the creation of acceptable time intervals for rodent cages removed from mechanical ventilation. These data will then facilitate the identification of equipment, interventions and actions that can be used when those durations might be exceeded.

Materials and Methods

Animals and microenvironment. This study was performed in compliance with a IACUC-approved protocol in an AAALAC-accredited facility supporting guidelines and policies governing the care and use of animals in research.[3] Colony mice and rats were SPF according to the site's exclusion list, which was based on FELASA recommendations.[11] Based on vendor exclusion methods, mice were free from mouse hepatitis virus, mouse parvoviruses (minute virus of mice), murine chapparovirus, ectromelia virus, K virus, polyoma virus, murine cytomegalovirus, epizootic diarrhea of infant mice virus, mouse thymic virus, lactate dehydrogenase elevating virus, mouse norovirus, Theiler murine encephalomyelitis virus, lymphocytic choriomeningitis virus, Sendai virus, pneumonia virus of mice, reovirus, Hantaan virus, mouse adenovirus, *Mycoplasma pulmonis*, *Bordetella bronchiseptica*, *Streptococcus pneumoniae*, *Pasteurella* spp. (*P. multocida*, *Rodentibacter pneumotropicus*, *R. heylii*), *Salmonella* spp., *Streptobacillus moniliformis*, *Filobacter rodentium*, *Corynebacterium kutscheri*, all *Helicobacter* spp., *Citrobacter rodentium*, *Campylobacter jejuni*, *Clostridium piliforme*, *Streptococcus zooepidemicus*, *Encephalitozoon cuniculi*, major gastrointestinal metazoan endoparasites (for example, *Syphacia* spp., *Aspiculuris* spp., *Rodentolepis* spp.), major ectoparasites (for example, *Myocoptes* spp., *Myobia* spp., *Radfordia* spp.), and major enteric protozoa (for example, *Coccidia*, *Giardia*, *Spironucleus*, *Eimeria* spp.). Rats were free of rat theilovirus, lymphocytic choriomeningitis virus, Sendai virus, pneumonia virus of mice, reovirus 3, Hantaan virus, mouse adenovirus, rat coronavirus/sialodacryoadenitis virus, rat parvoviruses (including rat parvovirus, rat minute virus, rat virus, and Toolan H1), infectious diarrhea of infant rats virus, *Mycoplasma pulmonis*, *Bordetella bronchiseptica*, *Streptococcus pneumoniae*, *Pasteurella* spp. (*P. multocida*, *Rodentibacter pneumotropicus*, *R. heylii*), *Salmonella* spp., *Streptobacillus moniliformis*, *Filobacter rodentium*, *Corynebacterium kutscheri*, all *Helicobacter* spp., *Clostridium piliforme*, *Pneumocystis carinii*, *Encephalitozoon cuniculi*, major gastrointestinal metazoan endoparasites (for example, *Syphacia* spp., *Aspiculuris* spp., *Rodentolepis* spp.), major ectoparasites (for example, *Myocoptes* spp., *Myobia* spp., *Radfordia* spp.), and major enteric protozoa (for example, *Coccidia*, *Giardia*, *Spironucleus*, *Eimeria* spp.).

Rodents were housed on IVC racks (Innorack IVC Mouse 3.5 or Innorack Tall IVC Rat, Innovive, San Diego, CA) in disposable 100% polyethylene terephthalate BPA-free plastic IVC caging with prefilled diced cellulose bedding (ALPHA-dri, Shepherd Specialty Papers, Watertown, TN).[6] Standard IVC lids (model MVX6 mouse IVC lid and model RVX7-AD rat tall IVC lid, Innovive) made of 100% polyethylene terephthalate BPA-free plastic and including a Reemay filter were used. C57BL/6J male mice (n = 50; age, 8 wk at arrival; weight, 21 to 26 g; The Jackson Laboratory, Bar Harbor, ME) were housed singly or at 4 or 5 per cage. Cages with 4 mice were evenly divided among experimental groups. Female virus-antibody–free Crl:CD(SD) rats (n = 27; weight, 400 to 500 g) were ordered from Charles River Laboratories (Wilmington, MA) and housed singly or in pairs. The strain and sex of rodents for the study reflected the highest census for each species inhouse and thus reflected our institutional conditions.

Mouse and rat IVC were set at positive pressure with 70 or 50 air changes per hour, respectively. They were housed on a 12:12-h light:dark cycle (fluorescent lighting, average of 250 lux, lights on, 0800). Temperatures were 70 to 74 °F (21 to 23 °C; set point, 72 °F [22 °C]) or, under thermoneutral conditions, 80 to 84 °F (27 to 29 °C; set point, 82 °F [28 °C] with a relative humidity of 30% to 70%. Only mice were housed at thermoneutral temperatures. Rooms were set at negative pressure and 10 to 15 air changes per hour. Chlorinated water (1 to 3 ppm; Aquavive, Innovive) in bottles and irradiated chow (Teklad 2920X, Envigo, Indianapolis, IN) were provided. After receipt, animals had at least 1 wk of acclimation prior to study. Enrichment included a site standard of 8 g crinkle paper (Bed-r'Nest, The Andersons Lab Bedding, Maunee, OH) for mice. Pair-housed rats received two 8-g pucks of crinkle paper, 2 nylon I Chews (Animal Specialties and Provisions, Quakertown, PA), and a Tall Rat Loft (R-LOFT, Innovive). Singly housed rodents received additional nesting material (Teklad 7979C, CS Diamond Twist, Envigo). All diet, bedding, and enrichment products were irradiated prior to receipt. Animals were monitored at least daily by staff.

Benchtop monitoring study design: mice. Our standard practice is to change cage bottoms of group-housed mice in IVC every 2 wk, with cage lid and accessories changed monthly. The cage bottoms, lids, and accessories of singly housed mice were changed every 28 d. Mice were weighed approximately 3 d before the start of the study. At the start of the study, cages were removed from the ventilated rack at 3 to 5 d after a scheduled full cage change, mimicking the middle of a standard change cycle, and were placed on a shelving cart (Metro, 5 × 537PG4, Wilkes-Barre, PA). For each cage, the original IVC lid was removed and replaced with an experimental lid. Cages with single- and group-housed mice were assigned to one of the following 6 groups (n = 3 cages for each lid type and temperature condition): standard temperature and sealed IVC lid (MVX6, Innovive), standard temperature and static lid (MS1 or MSX2, Innovive), standard temperature and unsealed (ajar) IVC lid (MVX6, Innovive), thermoneutral temperature with sealed IVC lid, thermoneutral temperature with static lid, and thermoneutral temperature with unsealed IVC lid (Figure 1). IVC lids were kept ajar by using a clean disposable wipe (WypAll, L40, Kimberly Clark, Roswell, GA) to hold the cage front open by approximately ½ in. to allow air exchange. Hourly monitoring began at 0800 when cages were removed and continued until 1500 or 1600 when placed back on the rack if intervention criteria were not met.

Benchtop monitoring study design: rats. Our standard practice is to change cage bottoms of IVC containing both singly and pair-housed rats weekly, with cage lids and accessories changed monthly. Rats were weighed approximately 3 d before the start of the study. To mimic the middle of the cage change cycle on study days, cages were removed from the ventilated rack at 3 to 5 d after a scheduled full cage change and placed on a shelving cart (5X537PG4, Metro, Wilkes-Barre, PA) On the study day, the original IVC lid from each cage was removed and replaced with an experimental lid. Cages with single- and group-housed rats were assigned to the following 3 groups (n = 3 cages per group): sealed IVC lid (RVX7-AD), sealed IVC lid with exhaust filter removed (this was done because a static lid is not available for the Tall IVC Rat Caging System), and unsealed IVC lid (Figure 2). Unsealed IVC lids were kept ajar by using a clean disposable wipe (WypAll, L40, Kimberly Clark) to hold

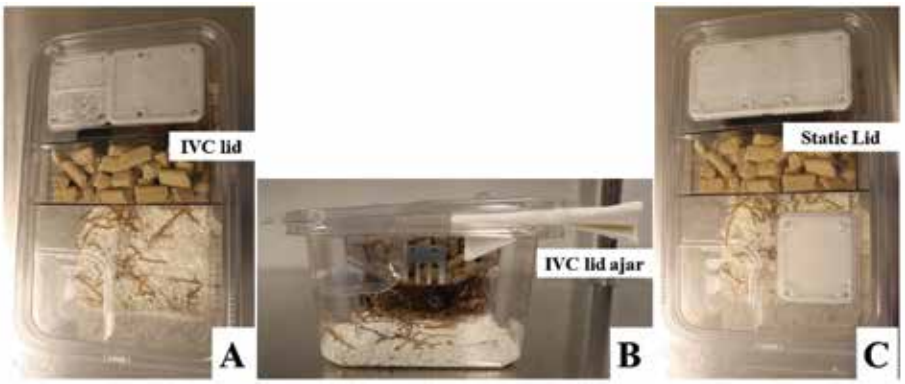

Figure 1. Representative images of mouse lid–cage conditions after removal from mechanical ventilation. (A) Mouse IVC lid. (B) Mouse IVC lid held ajar approximately 1/2 in. by using disposable cloth wipe. (C) Mouse static lid. Note: water bottles were removed temporarily and then restored after data acquisition.

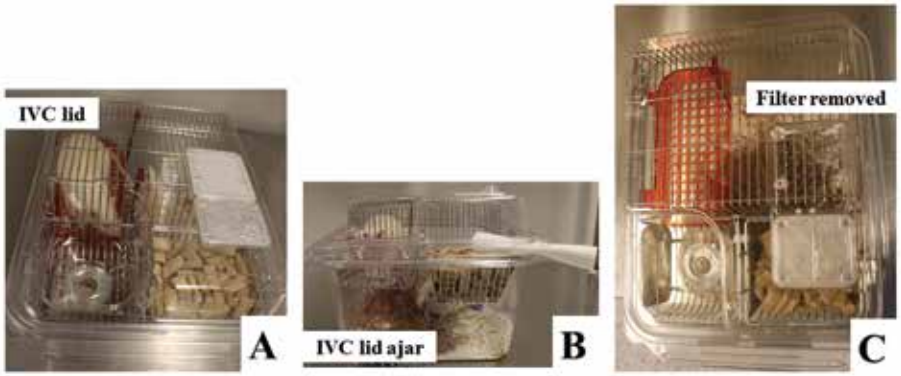

Figure 2. Representative images of rat lid–cage conditions after removal from mechanical ventilation: (A) Rat IVC lid. (B) Rat IVC lid held ajar approximately 1/2 in. by using disposable cloth wipe. (C) Rat IVC lid with filter removed. Note: water bottles were removed temporarily and then restored after data acquisition.

the cage front open approximately ½ in. to allow air exchange (Figure 2). Hourly monitoring began at 0800 when cages were removed and continued until 1500 or 1600 when placed back on the rack if intervention criteria were not met.

Microenvironmental testing. Environmental monitoring was performed using a multigas detection unit (Xam-8000, Draeger, Houston, TX) and colorimetric sensor (Small Animal Ammonia Sensor, Pacific Sentry, Redmond, WA) for NH_3 and CO_2 monitoring and a single-gas detection monitor and pump (Forensic Detectors, FD-90A-O2, Rolling Hills Estates, CA) for O_2 monitoring. All units were calibrated prior to use; data were recorded after gas readings had stabilized (within 15 s). Three trained personnel performed cageside welfare observations, with behavior noted (for example, alertness, quality of breathing, activity level) at each time point; if adverse clinical signs were observed, veterinary staff were alerted for confirmation and, if necessary, intervention.

Average room gas levels were: CO_2, 450 ppm; O_2, 21.0% vol; and NH_3, 0 ppm. Detector units sampled gas in the cages via nitrile tubing inserted through the water bottle access hole, with the tube placed at the estimated snout level of the rodent, approximately 4 cm from cage bottom (Figure 3). Colorimetric sensor tags were placed in the food hopper for exposure to cage air. Cages were changed and replaced on the rack when they met one of the following criteria: CO_2 levels exceeding 50,000 ppm, O_2 levels at or below 16%, NH_3 levels exceeding 50 ppm, condensation on the cage, and presence of clinical signs that included labored or shallow breathing and reduced activity. Regulatory thresholds for NH_3, O_2, and CO_2 levels are not available for rodents used in research; our criteria were selected with consideration of established human workplace recommendations and research that used similar test conditions.[4,13-16] A time-weighted average exposure limit of 50 ppm NH_3 is a National Institute for Occupational Safety and Health (NIOSH) recommendation.[14] An atmosphere with 4% (40,000 ppm) or more CO_2 is considered Immediately Dangerous to Life or Health for humans.[14] A previous study using the same cage system used the limit of detection of their multigas detection analyzer (50,000 ppm) as the threshold for CO_2, and we chose that threshold for comparison.[13] Hazardous atmospheres in the OSHA Confined Spaces in Construction standard include areas with 19.5% O_2 (normal ambient O_2 level is approximately 20.9%), and O_2 levels above 15% are considered safe for most healthy, fit persons as long as they are sedentary.[4,14]

Statistical analysis. Data were analyzed by using R (version 4.0.5, R Foundation for Statistical Computing, Vienna, Austria). Data are presented as mean ± SD unless otherwise stated. A longitudinal mixed-effects model with a first-order autoregressive covariance structure was used to compare the O_2 and CO_2 levels between lid-type groups over time for singly housed mice, group-housed mice, singly housed mice at thermoneutral temperatures, group-housed mice at thermoneutral temperatures, singly housed rats, and pair-housed rats. We only used data collected at 1, 4, and 7 or 8 h after the removal of IVC from mechanical ventilation because values were relatively linear relative to measurements at 2-3 h and 5-6 h during this period. A Pearson correlation coefficient was computed to investigate the linear relationship between O_2 and CO_2 levels. A *P* value of

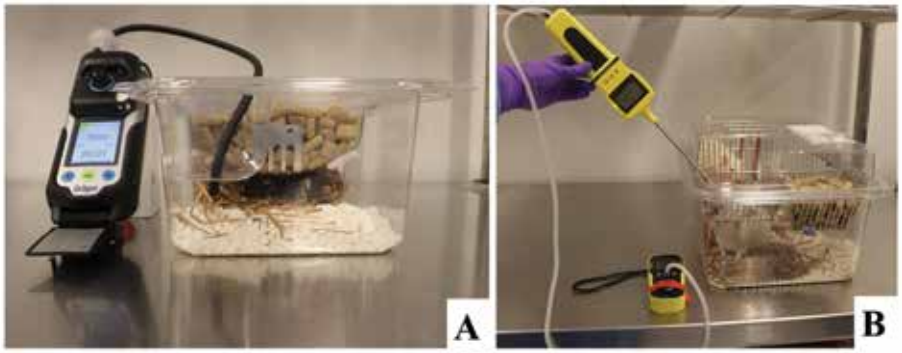

Figure 3. Representative images of gas sampling procedures for (A) mouse cage with CO_2-NH_3 meter and (B) rat cage with O_2 meter. Note: water bottles removed were temporarily and then restored after data acquisition.

less than 0.05 was considered as statistically significant. Rodent body weights were compared using one-way ANOVA (Prism, version 9.0.0, GraphPad Software, San Diego, CA).

Results

Microenvironmental conditions of mouse IVC with 3 lid types after removal from ventilation at standard and thermoneutral temperatures. Mean body weights of group-housed mice did not differ among groups nor did the weights of singly housed mice ($F_{2,39} = 0.16$, $P = 0.84$ and $F_{2,6} = 0.66$, $P = 0.55$, respectively). O_2 (Table 1) and CO_2 (Table 2) levels showed an almost perfect inverse linear correlation over time ($r_7 = –0.99$; $P \leq 0.05$; Figure 4). NH_3 was not detected in any cages throughout the study.

In cages with group-housed mice under standard temperature conditions (set point of 72°F), at 1, 4, and 8 h after removal from ventilation, O_2 levels in cages with sealed IVC lids were lower than those of with static and unsealed lids ($P \leq 0.0001$ at 1 and 4 h; $P \leq 0.01$ and $P \leq 0.05$, respectively, at 8 h). O_2 levels in IVC with group-housed mouse were not different between static and unsealed (partially ajar) lids at 1, 4, and 8 h ($P = 0.41$, $P = 0.96$, and $P = 0.46$, respectively). CO_2 levels in cages with sealed IVC lids were significantly higher than those of IVC with unsealed lids or static lids ($P \leq 0.0001$ and $P \leq 0.001$ at 1 h; $P \leq 0.001$ for both at 4 h; and $P \leq 0.01$ and $P \leq 0.05$ at 8h). At 1 h after removal of IVC from ventilation, 1 of 3 group-housing IVC lid cages met study removal criteria at standard temperatures ($CO_2 < 50,000$ ppm; clinical signs of increased respiratory rate and effort).

In cages with singly housed mice at the thermoneutral temperature (set point of 82°F), CO_2 levels did not differ between IVC with static lids or unsealed lids at 1, 4, or 8 h ($P = 0.55$, $P = 0.97$, and $P = 0.62$, respectively). Changes in O_2 and CO_2 were also not significantly different over time for single-occupancy cages, and no cages met the criteria for removal from study (Tables 1 and 2).

Under thermoneutral conditions, O_2 levels for group-housed mice in IVC with sealed lids were significantly lower than those of mouse cages with static or unsealed lids ($P \leq 0.0001$ and $P = 0.01$ at 1 h:, $P \leq 0.001$ and $P \leq 0.01$ at 4 h; and $P \leq 0.05$ for both at 8 h). O_2 levels did not differ between IVC with unsealed or static lids ($P = 0.08$ at 1 h, $P = 0.15$ at 4 h, and $P = 0.69$ at 8 h). CO_2 levels in IVC with sealed lids were significantly higher than those of cages with static or unsealed lids ($P \leq 0.0001$ and $P \leq 0.01$ respectively at both 1 and 4 h; $P < 0.01$ and $P < 0.05$ at 8 h). At 2 h, 2 of the 3 group-housed IVC with sealed lids met study removal criteria ($CO_2 > 50,000$ ppm; Figure 4 C and D). Levels of O_2 and CO_2 had similar significance values over time for single-occupancy cages, except that O_2 levels in IVC with static lids were significantly higher than those in IVC with unsealed lids at 1 and 4 h (both $P \leq 0.05$; Tables 1 and 2) but not at 8 h ($P = 0.15$). None of the single-occupancy cages met the criteria for removal from study under thermoneutral conditions.

Microenvironmental conditions of rat IVC cages with 3 lid types after removal from ventilation at standard temperature. Body weights of pair-housed and singly-housed rats were not different among experimental groups ($F_{2,15} = 0.09$, $P = 0.91$ and $F_{2,6} = 0.41$, $P = 0.68$, respectively). Figure 5 shows CO_2 and O_2 levels over time for rat cages after removal from mechanical ventilation. Tables 3 and 4 show hourly O_2 and CO_2 levels for rat cages at standard temperature (set point of 72°F). NH_3 was not detected in any rat IVC throughout the study. At 1 h, all 3 pair-housed IVC with sealed lids and 2 pair-housed IVC with exhaust filters removed met study removal criteria ($CO_2 > 50,000$ ppm; humidity from 70.1% to 74.6%, clinical signs of lethargy and increased respiration). Pair-housed IVC with sealed lids had significantly higher CO_2 levels than did those with exhaust filters removed or unsealed lids at 1 h ($P \leq 0.05$ and $P \leq 0.01$, respectively). In addition, O_2 levels at 1 h were significantly lower in pair-housed IVC with sealed lids as compared with those with exhaust filters removed or unsealed lids ($P \leq 0.05$ and $P \leq 0.01$, respectively). Furthermore, condensation was present on the walls of the affected IVC. At 2 h, IVC with a single rat and sealed lid had significantly higher CO_2 levels than did those with exhaust filters removed or unsealed lids ($P \leq 0.0001$ and $P \leq 0.01$). In addition, IVC with single rats had significantly lower O_2 levels with sealed lids compared with those having exhaust filters removed or unsealed lids ($P \leq 0.01$ and $P \leq 0.0001$, respectively). By 3 h, all 3 single-housed IVC with sealed lids met study removal criteria ($CO_2 > 50,000$ ppm; clinical signs of lethargy and increased respiration). IVC with pair- and singly housed rats with unsealed lids or exhaust filters removed did not meet intervention criteria through 7 h (end of study).

Discussion

The microenvironment of disposable IVC with sealed lids was acceptable for singly housed mice for at least as long as 8 h and for group-housed mice ($n = 4$ or 5) for 1 h at standard temperatures and for 2 h at thermoneutral temperatures. Clinical observations consistent with hypoxia or hypercapnia were observed for one of the group-housed IVC with sealed lids that had > 40,000 ppm CO_2 and 16.4% O_2 at 1 h standard temperature, and for 2 of the group-housed sealed IVC cages at greater than 40,000 ppm CO_2 and 16.3% O_2 at 2 h at thermoneutral temperature. Under the standard temperatures, these findings are consistent with another study of group-housed mice in this cage type with different lids.[13] We speculate that the more frequent clinical signs and greater CO_2 buildup between the

Table 1. CO_2 levels (ppm; mean ± 1 SD; $n = 3$ unless otherwise indicated in parentheses) in group- or single-occupied mouse disposable IVC cages at standard or thermoneutral conditions with 3 lid types

		Time (h) after removal from mechanical ventilation								
Occupancy	Lid	0	1	2	3	4	5	6	7	8
Standard temperature condition										
Group	Sealed IVC	6,600 ± 265	38,750 ± 4,596[c,h]	32,500 ± 6,364 (2)	29,300 ± 6,647 (2)	26,600 ± 3,394[c,g] (2)	25,500 ± 3,536 (2)	25,500 ± 0 (2)	22,800 ± 3,111 (2)	26,150 ± 3,323[a,f] (2)
	Unsealed IVC	4,850 ± 1011	6,500 ± 854	7,417 ± 2398	4,867 ± 2,098	3,500 ± 1,838	5,600 ± 2,425	8,733 ± 5,270	7,217 ± 4,762	9,200 ± 5,923
	Static	6,066 ± 850	11,000 ± 2,107	8,833 ± 907	6,367 ± 907	6,233 ± 2,318	6,267 ± 2,023	5,700 ± 2,425	5,200 ± 2,084	5,233 ± 1,750
Single	Sealed IVC	1,350 ± 409	9,367 ± 802[d,l]	8,900 ± 1,411	8,500 ± 2,128	9,367 ± 1,193[d,l]	9,433 ± 451	7,633 ± 1,270	7,500 ± 265	8,333 ± 1,115[c,l]
	Unsealed IVC	917 ± 76	2,167 ± 189[i]	1,650 ± 853	1,533 ± 1,107	1,167 ± 333 †	1,267 ± 306	1,933 ± 1,504	2,150 ± 1,602	1,717 ± 715
	Static	983 ± 18	4,183 ± 505	5,355 ± 580	3,233 ± 486	3,200 ± 250	3,333 ± 577	3,600 ± 726	2,967 ± 236	2,950 ± 328
Thermoneutral temperature condition										
Group	Sealed IVC	3,617 ± 475	39,333 ± 5,058[d,f]	42,000 ± 7,071	30,500 (1)	27,500[d,j] (1)	22,000 (1)	22,400 (1)	23,200 (1)	21,600[b,i] (1)
	Unsealed IVC	3,583 ± 839	19,767 ± 7,959	21,200 ± 2,946	13,067 ± 1,815	13,133 ± 2,831	12,667 ± 2,082	16,533 ± 6,133	9,300 ± 5,556	7,700 ± 4,500
	Static	3,300 ± 361	11,733 ± 1,405	10,133 ± 1,604	8,200 ± 100	7,433 ± 839	7,367 ± 635	6,333 ± 1,102	6,733 ± 1,250	6,633 ± 115
Single	Sealed IVC	1,117 ± 247	9,167 ± 1,290[b]	10,000 ± 1,778	9,200 ± 1,200	7,867 ± 1,350[b,i]	7,933 ± 902	7,800 ± 1,769	7,400 ± 1,039	7,500 ± 1,997[b]
	Unsealed IVC	1,033 ± 189	6,267 ± 874	5,133 ± 1,106	5,067 ± 902	5,217 ± 1,130	5,183 ± 881	4,183 ± 2,177	3,717 ± 2,326	4,167 ± 2,532
	Static	867 ± 202	3,917 ± 382	2,250 ± 1,106	1,767 ± 751	1,867 ± 982	1,833 ± 1,102	2,400 ± 1,253	1,567 ± 861	2,333 ± 1,882

Statistical analysis between lid types at the same time point was performed for the 1-, 4-, and 8-h time points.
[a]$P < 0.05$, sealed IVC compared with static lid
[b]$P < 0.01$, sealed IVC compared with static lid
[c]$P < 0.001$, sealed IVC compared with static lid
[d]$P < 0.0001$, sealed IVC compared with static lid
[e]$P < 0.05$, sealed IVC compared with unsealed lid
[f]$P < 0.01$, sealed IVC compared with unsealed lid
[g]$P < 0.001$, sealed IVC compared with unsealed lid
[h]$P < 0.0001$, sealed IVC compared with unsealed lid
[i]$P < 0.05$, unsealed lid compared with static lid
[j]$P < 0.01$, unsealed lid compared with static lid
[k]$P < 0.001$, unsealed lid compared with static lid
[l]$P < 0.0001$, unsealed lid compared with static lid

2 temperature conditions is in part due to lower spontaneous activity under thermoneutral conditions.[17] For planned off-rack use longer than these durations, we recommend allowing increased air exchange by leaving the lid partially ajar while mice are monitored or by using a static lid. The microenvironment in disposable IVC with sealed lids was acceptable for a maximum of 1 h for pair-housed rats and for as long as 3 h for singly housed rats at standard temperatures. Clinical signs consistent with hypoxia or hypercapnia were observed in all 3 pair-housed rats in sealed IVC cages at greater than 40,000 ppm CO_2 and 15.1% O_2 at 1 h and in all 3 singly housed rats in sealed-lid IVC at greater than 40,000 ppm CO_2 and 17.3% O_2 at 3 h. Two of the 3 pair-housed rats with filters removed cages had greater than 40,000 ppm CO_2 and 16.2% O_2 at 1 h. Therefore, removing the exhaust filter from the IVC lid did not appear to allow adequate air circulation. To our knowledge, this report is the first to describe conditions for unventilated disposable IVC housing in rats.

Consistent with our observations in mice, CO_2 levels of 40,000 ppm or greater were associated with the onset of clinical signs in rats and could provide updated criteria for intervention. After the identification and removal of the clinically affected pair-occupied IVC with exhaust filters removed, the remaining cage in that cohort showed reductions and stabilization of CO_2 levels over time. We suspect that in cages that did not reach the intervention criteria, the activity of the rodents decreased and helped alleviate further exacerbation of conditions and stabilize air exchange. Decreased activity could occur due to acclimation (reducing stress) or stabilization of air quality over time; alternatively, decreased activity could have reflected lethargy due to hypercarbia or hypoxia. However, we did not observe any signs of lethargy in these rats; therefore, coordination of activity and air exchange—rather than worsening of cage conditions—was the most likely explanation.

One preventative measure that effectively promoted air exchange was to leave the lid partially ajar with stainless steel grid intact, providing that animals were monitored. In addition, when the lids are used acutely, for example as at the end of a study, we have observed that cutting of both the plastic around the exhaust area of the lid and the filter itself was

Table 2. O_2 levels (%; mean ± 1 SD; n = 3 unless otherwise indicated in parentheses) in group- or single-occupied mouse disposable IVC at standard or thermoneutral conditions with various lid types

		Time (h) after removal from mechanical ventilation								
Housing	Lid	0	1	2	3	4	5	6	7	8
Standard temperature condition										
Group	Sealed IVC	20.5 ± 0.1	17 ± 0.6[d,l]	17.8 ± 0.8 (2)	18.2 ± 0.8 (2)	18.8 ± 0.6[d,l] (2)	18.2 ± 0.3 (2)	18.6 ± 0.1 (2)	18.8 ± 0.2 (2)	18.5 ± 0.3[b,i] (2)
	Unsealed IVC	20.6 ± 0.1	20.5 ± 0.1	20.3 ± 0.2	20.7 ± 0.3	20.6 ± 0.2	20.6 ± 0.2	20.1 ± 0.6	20.2 ± 0.6	20.1 ± 0.6
	Static	20.4 ± 0.1	20 ± 0.2	20.2 ± 0	20.4 ± 0.1	20.4 ± 0.2	20.4 ± 0.2	20.5 ± 0.3	20.6 ± 0.1	20.6 ± 0.1
Single	Sealed IVC	20.9 ± 0	20.1 ± 0.1[c,l]	20.2 ± 0	20.2 ± 0.2	20.1 ± 0.2[d,l]	20.1 ± 0.1	20.3 ± 0.2	20.2 ± 0.1	20.2 ± 0.1[c,k]
	Unsealed IVC	20.9 ± 0	20.9 ± 0	20.9 ± 0	20.9 ± 0.1	20.8 ± 0.1	20.8 ± 0	20.8 ± 0.1	20.8 ± 0.2	20.8 ± 0.2
	Static	20.9 ± 0	20.7 ± 0.1	20.7 ± 0.1	20.7 ± 0	20.7 ± 0	20.8 ± 0.1	20.7 ± 0.1	20.7 ± 0.1	20.7 ± 0.1
Thermoneutral condition										
Group	Unsealed IVC	20.8 ± 0.1	16.9 ± 0.6[d,j]	16.8 ± 0.8	18.3 (1)	18.1 (1)[c,j]	19.3 (1)	19.3 (1)	19.2 (1)	19.5 (1)[a,i]
	Ajar IVC	20.8 ± 0.1	19.2 ± 0.9	19.3 ± 0.3	19.9 ± 0.1	20.0 ± 0.2	19.9 ± 0.1	19.7 ± 0.7	20.5 ± 0.5	20.5 ± 0.4
	Static	20.9 ± 0	20.1 ± 0.1	20.3 ± 0.2	20.4 ± 0.1	20.6 ± 0.1	20.6 ± 0.1	20.7 ± 0.1	20.7 ± 0.1	20.6 ± 0
Single	Unsealed IVC	20.9 ± 0	20.3 ± 0.2[c,j]	20.3 ± 0.2	20.4 ± 0.1	20.6 ± 0.1[d,j]	20.6 ± 0	20.6 ± 0.1	20.6 ± 0.1	20.6 ± 0.1[a]
	Ajar IVC	20.9 ± 0	20.7 ± 0.1[e]	20.7 ± 0.1	20.7 ± 0	20.8 ± 0.1[e]	20.8 ± 0.1	20.9 ± 0.2	20.8 ± 0.1	20.9 ± 0.2
	Static	21.1 ± 0	21.0 ± 0.1	21.0 ± 0.1	20.9 ± 0	21.0 ± 0.1	20.9 ± 0.1	21.0 ± 0.1	21.0 ± 0.1	21.0 ± 0.1

Statistical analysis between lid types at the same time point was performed for the 1-, 4-, and 8-h time points.
[a]$P < 0.05$, sealed IVC compared with static lid
[b]$P < 0.01$, sealed IVC compared with static lid
[c]$P < 0.001$, sealed IVC compared with static lid
[d]$P < 0.0001$, sealed IVC compared with static lid
[e]$P < 0.05$, sealed IVC compared with unsealed lid
[f]$P < 0.01$, sealed IVC compared with unsealed lid
[g]$P < 0.001$, sealed IVC compared with unsealed lid
[h]$P < 0.0001$, sealed IVC compared with unsealed lid
[i]$P < 0.05$, unsealed lid compared with static lid
[j]$P < 0.01$, unsealed lid compared with static lid
[k]$P < 0.001$, unsealed lid compared with static lid
[l]$P < 0.0001$, unsealed lid compared with static lid

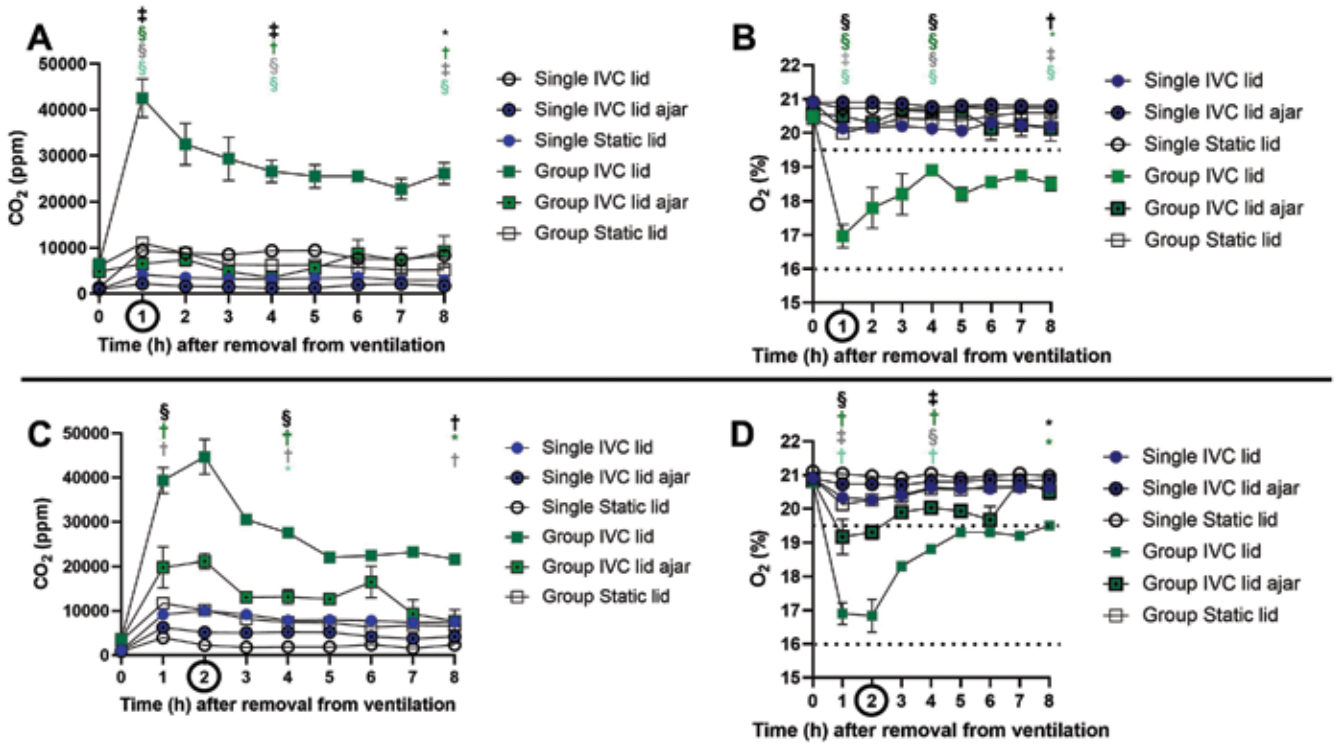

Figure 4. (A and C) CO_2 and (B and D) O_2 conditions in mouse caging after removal from ventilation over time according to cage occupancy and lid type at standard temperatures (A and B) and thermoneutral temperatures (C and D). Data are shown as mean ± SEM. Circled numbers indicate the observation of clinical signs and removal of group IVC lid cages from study at that time point. Colors indicate significant differences between conditions (black (group) and gray (single), sealed IVC lid compared with static lid; green (group) and light green (single), sealed IVC lid compared with unsealed IVC lid; blue (group) and light blue (single), unsealed IVC lid compared with static lid): *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; §, $P < 0.0001$.

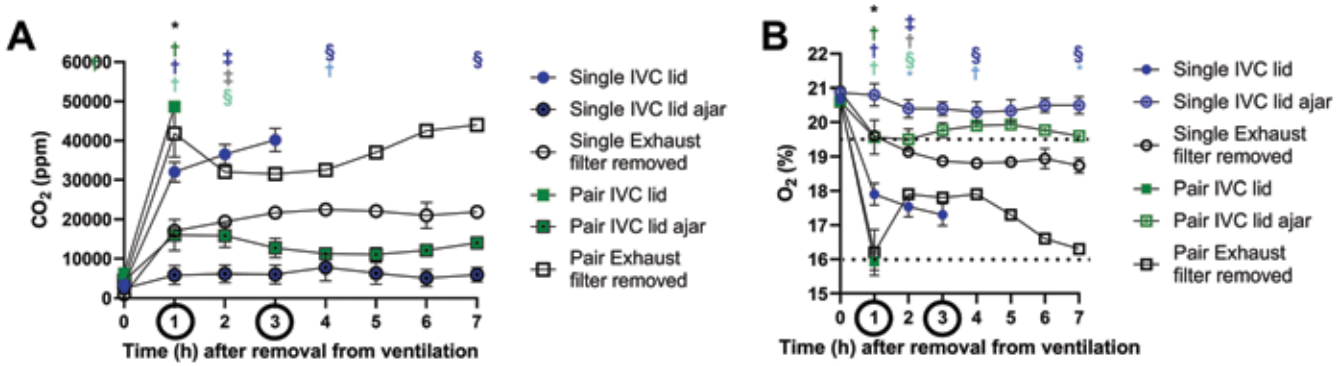

Figure 5. (A) CO_2 and (B) O_2 conditions of rat caging after removal from ventilation according to cage occupancy and lid type at standard temperatures. Data are shown as mean ± SEM. Circled numbers indicate the observation of clinical signs and removal of cages that were paired housing with IVC lid, paired housing with exhaust filter removed, and single housing with IVC lid cages at that time point. Colors indicate significant differences between conditions (black (pair) and gray (single), sealed IVC lid compared with exhaust filter removed; green (pair) and light green (single), sealed IVC lid compared with unsealed IVC lid; blue (pair) and light blue (single), exhaust filter removed compared with unsealed IVC lid): *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; §, $P < 0.0001$.

Table 3. CO_2 levels (ppm; mean ± 1 SD; $n = 3$ unless otherwise indicated in parentheses) in pair- or single-occupied rat disposable IVC cages at standard temperature with 3 lid types

		Time (h) after removal from mechanical ventilation							
Occupancy	Lid	0	1	2	3	4	5	6	7
Pair	Sealed IVC	6,233 ± 2,050	48,000 ± 2,828[a,f]	NA	NA	NA	NA	NA	NA
	Filter removed	4,400 ± 1,900	41,833 ± 10,396[j]	32,000 (1)[k]	31,500 (1)	32,500[l] (1)	37,000 (1)	42,500 (1)	44,000[l] (1)
	Unsealed IVC	4,433 ± 1,710	16,033 ± 6,873	15,867 ± 5,217	12,733 ± 4,277	11,267 ± 2,344	11,067 ± 3,002	12,133 ± 1,206	14,000 ± 1,744
Single	Sealed IVC	3,467 ± 2,354	32,000 ± 4,359[j]	36,500 ± 4,359[c,h]	40,167 ± 5,132	NA	NA	NA	NA
	Filter removed	967 ± 115	17,067 ± 833	19,400 ± 1,200[i]	21,733 ± 1,677	22,533 ± 2,403[j]	22,133 ± 1,901	21,033 ± 5,719	21,933 ± 1,514[j]
	Unsealed IVC	2,483 ± 1,145	5,883 ± 4,114	6,150 ± 3,887	6,033 ± 4,045	7,767 ± 2,344	6,317 ± 4,750	5,100 ± 3,646	5,967 ± 3,062

Statistical analysis between lid types at the same time point was performed for the 1-, 4-, and 7-h time points.
NA, not applicable
[a]$P < 0.05$, sealed IVC compared with exhaust filter removed
[b]$P < 0.01$, sealed IVC compared with exhaust filter removed
[c]$P < 0.001$, sealed IVC compared with exhaust filter removed
[d]$P < 0.0001$, sealed IVC compared with exhaust filter removed
[e]$P < 0.05$, sealed IVC compared with unsealed lid
[f]$P < 0.01$, sealed IVC compared with unsealed lid
[g]$P < 0.001$, sealed IVC compared with unsealed lid
[h]$P < 0.0001$, sealed IVC compared with unsealed lid
[i]$P < 0.05$, unsealed lid compared with exhaust filter removed
[j]$P < 0.01$, unsealed lid compared with exhaust filter removed
[k]$P < 0.001$, unsealed lid compared with exhaust filter removed
[l]$P < 0.0001$, unsealed lid compared with exhaust filter removed

effective. However, keeping the lid unsealed for occupied cages introduces biosecurity risks, allergen concerns, and the possibility of animal escape or injury; thus these practices should be reviewed with researchers and staff prior to implementation to determine whether they are appropriate. Literature from the vendor suggests that during a power failure, the cage-level exhaust filters protect a vent at the rear of the cage to ensure mice with MVX6 lids and rats with RVX7 lids will survive for at least 24 and 48 h, respectively; of course, these estimates depend on the size and number of animals in the cage.[6] Although CO_2 and O_2 levels were stable for as long as 8 h in singly housed mice and 7 h in singly housed rats, additional work is needed to determine whether levels equilibrate and maintain sufficient air exchange beyond these time points, especially for group-occupied cages, thus avoiding lethal conditions in emergency situations. Based on the findings in this study, our emergency actions are to remove lids for ventilation, to change cages, or to take other action as directed by veterinary staff when power loss is sustained for over 12 h. For any sealed off-ventilation IVC in which animals show clinical signs or cageside observations indicate a hypercapnic or hypoxic environment, the cage should be changed and veterinary staff alerted for examination and follow up.

Both hypoxic and hypercarbic conditions can impair the cardiovascular, respiratory and metabolic physiology of rodents and, in turn, affect research outcomes.[9,18] The effects of hypercarbia in rodents have been summarized.[7] Although the conditions in our study were not as severe as in some experimental settings, we infer from our clinical observations (for example, increased respiratory rate and effort, reduced activity) that compensatory physiologic reactions occurred and thus have the potential to alter outcomes despite recovery. Long-term exposure to hypoxia inhibits tumor progression in lung cancer models in mice and rats.[19] Both acute and intermittent hypoxia exposure can affect the systemic and pulmonary circulations of C57BL/6J mice.[3] The specific response to some conditions might depend on the individual rodent, including its strain and sex; thus, in general, extended periods without adequate air exchange should be avoided.[1,2,20]

Table 4. Oxygen levels (%; mean ± 1 SD; n = 3 unless otherwise indicated in parentheses) in pair- or single-occupied rat disposable IVC at standard temperature with 3 lid types

		Time (h)							
Occupancy	Lid	0	1	2	3	4	5	6	7
Pair	Sealed IVC	20.6 ± 0.2	15.9 ± 0.5[a,f]	NA	NA	NA	NA	NA	NA
	Filter removed	20.6 ± 0.1	16.2 ± 1.2[j]	17.9 (1)[k]	17.8 (1)	17.9[l] (1)	17.3 (1)	16.6 (1)	16.3[l] (1)
	Unsealed IVC	20.6 ± 0.1	19.6 ± 0.9	19.5 ± 0.5	19.8 ± 0.4	19.9 ± 0.3	19.9 ± 0.2	19.8 ± 0.1	19.6 ± 0.3
Single	Sealed IVC	20.7 ± 0.2	17.9 ± 0.6[f]	17.5 ± 0.5[b,h]	17.3 ± 0.6	NA	NA	NA	NA
	Filter removed	20.9 ± 0	19.6 ± 0.1	19.1 ± 0.1[i]	18.9 ± 0.2	18.8 ± 0.2[j]	18.8 ± 0.2	18.9 ± 0.6	18.7 ± 0.4[j]
	Unsealed IVC	20.9 ± 0.1	20.8 ± 0.6	20.4 ± 0.5	20.4 ± 0.3	20.3 ± 0.5	20.3 ± 0.6	20.5 ± 0.4	20.5 ± 0.4

Statistical analysis between lid types at the same time point was performed for the 1-, 4-, and 7-h time points.
NA, not applicable
[a]$P < 0.05$, sealed IVC compared with exhaust filter removed
[b]$P < 0.01$, sealed IVC compared with exhaust filter removed
[c]$P < 0.001$, sealed IVC compared with exhaust filter removed
[d]$P < 0.0001$, sealed IVC compared with exhaust filter removed
[e]$P < 0.05$, sealed IVC compared with unsealed lid
[f]$P < 0.01$, sealed IVC compared with unsealed lid
[g]$P < 0.001$, sealed IVC compared with unsealed lid
[h]$P < 0.0001$, sealed IVC compared with unsealed lid
[i]$P < 0.05$, unsealed lid compared with exhaust filter removed
[j]$P < 0.01$, unsealed lid compared with exhaust filter removed
[k]$P < 0.001$, unsealed lid compared with exhaust filter removed
[l]$P < 0.0001$, unsealed lid compared with exhaust filter removed

Additional studies are warranted to determine any downstream effects of these findings on rodents' overall health and wellbeing and any research implications. Our study was limited due to using only 3 cages per condition and to not examining other variables, such as other sexes, strains, ages, mouse housing density (for example, 2 or 3 per cage), and body condition types that may exhibit differences in tolerance to microenvironmental conditions. Further investigation is also needed to determine whether opening and closing lids for a specified duration at regular intervals would permit sufficient ventilation to avoid undesirable conditions.

At our site, common areas of research conduct in which rodents might be housed without ventilation for extended periods include preparation for and recovery from surgery, imaging, behavioral testing, study termination, and training. As examples, anesthetic recovery after an imaging event could be prolonged or complicated after an animal is placed in a cage with inadequate ventilation, and prolonged removal from ventilation for cages awaiting experimental termination may create a stressful environment for animals prior to euthanasia. For cages at risk, staff and researchers should bring cages to experimental rooms by group or in tiers to avoid inappropriate environmental conditions, use static lids for mouse cages, and set lids either partially ajar or remove the filter and associated plastic of the exhaust supply port for air exchange in rat cages. Rodents that develop signs or cage conditions suggestive of a hypercarbic or hypoxic environment should receive prompt intervention. Although they appear to recover uneventfully, we recommend reacclimation of as long as 3 d before experimental termination or manipulation to allow stabilization and ideally to avoid potential research interference.[7,8] In addition, we advise that unventilated time be limited to the shortest period needed to perform the work. With this guidance, we hope to alert scientists and the rest of the animal research community to these potential changes in microenvironment and provide recommendations to mitigate effects on the animals and ongoing research.

Acknowledgments

We thank Stephen Baker, Michael Wadanoli, and Chandra Williams for manuscript review; John Stansfield for statistical assistance and review; Orrin James and Madison Traystman for technical support; and Dora Andrade, Yessica Flores, and Francisca Rodriguez for husbandry support.

References

1. **Améndola L, Weary DM.** 2019. Evidence for consistent individual differences in rat sensitivity to carbon dioxide. PLoS One **14:**e0215808. https://doi.org/10.1371/journal.pone.0215808.
2. **Arias-Reyes C, Soliz J, Joseph V.** 2021. Mice and rats display different ventilatory, hematological, and metabolic features of acclimatization to hypoxia. Front Physiol **12:**647822. https://doi.org/10.3389/fphys.2021.647822.
3. **Campen MJ, Shimoda LA, O'Donnel CP.** 2005. Acute and chronic cardiovascular effects of intermittent hypoxia in C57BL/6J mice. J Appl Physiol **99**:2028–2035. https://doi.org/10.1152/japplphysiol.00411.2005.
4. **Health and Safety Executive.** [Internet]. 2018. A health risk assessment of working in hypoxic atmospheres. RR1137. [Cited 8 March 2022]. Available at: https://www.hse.gov.uk/research/rrhtm/rr1137.htm.
5. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
6. **Innovive Disposable Cages.** [Internet]. IVC racks and static rodent cage housing for mice and rats for research laboratory facilities FAQ. [Cited 8 March 2022]. Available at: https://www.innovive.com/our-system/faq.html
7. **Krohn TC, Hansen AK.** 2000. The effects of and tolerance for carbon dioxide in relation to recent developments in laboratory animal housing. Scand J Anim Sci **27:**173–181. https://doi.org/10.23675/sjlas.v27i3.30.

8. **Krohn TC, Hansen AK.** 2002. Carbon dioxide concentrations in unventilated IVC cages. Lab Anim **36:**209–212. https://doi.org/10.1258/0023677021912361.
9. **Drew KL, Harris MB, LaManna JC, Smith MA, Zhu XW, Ma YL.** 2004. Hypoxia tolerance in mammalian heterotherms. J Exp Biol **207:**3155–3162. https://doi.org/10.1242/jeb.01114.
10. **Levy DRM, Flores R, Garcia GE, Craig SL, Jensen VB.** 2018. Effects of extruded compared with pelleted diets on laboratory mice housed in individually ventilated cages and environment. J Am Assoc Lab Anim Sci **57:**686–694. https://doi.org/10.30802/AALAS-JAALAS-17-000110.
11. **Mähler M, Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning K, Raspa M.** 2014. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. Lab Anim **48:**178–192. https://doi.org/10.1177/0023677213516312.
12. **Tataryn NM, Buckmaster CA, Schwiebert RS, Swennes AG.** 2021. Comparison of four beddings for ammonia control in individually ventilated mouse cages. J Am Assoc Lab Anim Sci **60:**37–43. https://doi.org/10.30802/AALAS-JAALAS-20-000051.
13. **Nagamine CM, Long CT, McKeon GP, Felt SA.** 2012. Carbon dioxide and oxygen levels in disposable individually ventilated cages after removal from mechanical ventilation. J Am Assoc Lab Anim Sci **51:**155–161.
14. **National Institute for Occupational Safety & Health.** [Internet]. 2020. NIOSH pocket guide to chemical hazards. [Cited March 9 2022]. Available at: https://www.cdc.gov/niosh/npg/default.html
15. **Silverman J, Bays DW, Baker SP.** 2008. Ammonia and carbon dioxide concentrations in disposable and reusable ventilated mouse cages. J Am Assoc Lab Anim Sci **47:**57–62.
16. **Silverman J, Bays DW, Baker SP.** 2009. Ammonia and carbon dioxide concentrations in disposable and reusable static mouse cages. Lab Anim **38:**16–23. https://doi.org/10.1038/laban0109-16.
17. **Swoap SJ, Overton JM, Garber G.** 2004. Effect of ambient temperature on cardiovascular parameters in rats and mice: A comparative approach. Am J Physiol Regul Integr Comp Physiol **287:**R391–R396. https://doi.org/10.1152/ajpregu.00731.2003.
18. **Tolstun DA, Knyazer A, Tushynka TV, Dubiley TA, Bezrukov VV, Fraifeld VE, Muradian KK.** 2020. Metabolic remodeling of mice by hypoxic-hypercapnic environment: Imitating the naked mole rat. Biogerontology **21:**143–153. https://doi.org/10.1007/s10522-019-09848-9.
19. **Yu L, Hales CA.** 2011. Long-term exposure to hypoxia inhibits tumor progression of lung cancer in rats and mice. BMC Cancer **11:**331. https://doi.org/10.1186/1471-2407-11-331.
20. **Zwemer CF, Song MY, Carello KA, D'Alecy LG.** 2007. Strain differences in response to acute hypoxia: CD1 versus C57BL/6J mice. J Appl Physiol **102:**286–293. https://doi.org/10.1152/japplphysiol.00536.2006.