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Tolerance of Atmospheric Ammonia by Laboratory Mice

A.R. Green, NSF Phd Graduate Fellow

Agricultural and Biosystems Engineering, Iowa State University, angelag@iastate.edu.

C.M. Wathes, Professor

Royal Veterinary College, University of London, cmwathes@rvc.ac.uk.

T.G.M. Demmers, PhD

Royal Veterinary College, University of London, tdemmers@rvc.ac.uk.

J. MacArthur-Clark

Pfizer, judy.macarthurclark@pfizer.com

H. Xin, Professor

Agricultural and Biosystems Engineering, Iowa State University, hxin@iastate.edu.

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Abstract. *A novel preference chamber with four inter-connected compartments was designed and built to test the tolerance of atmospheric ammonia by laboratory mice. The preference chamber incorporated a novel tracking system using an infra-red sensor at each end of each tunnel, which monitored all journeys through the tunnels and their direction. An experiment was successfully undertaken with four batches, each of four mice. Each batch was housed in the chamber for 4 days and given the choice between ammonia concentrations of nominally 0, 25, 50 and 100 ppm after initial familiarization. The results showed that there were two motivations acting on mouse behavior. The mice made extensive use of the whole chamber once they had been trained to use the tunnels, at least 2000 movements between compartments for each group over 48 h. The mice clearly preferred to be in the upper two compartments of the top tier of the chamber rather than in the lower compartments. The mice did not exhibit a clear preference for or aversion to ammonia, which*

implies that their short- term tolerance of ammonia at potentially noxious concentrations may not be in their long-term interest.

Keywords. Preference test, chamber, aversive, ammonia, behavior, *Mus musculus*.

Introduction

A recent review (Latham and Mason, 2004) has highlighted the influence of the environment on the health, behavior and welfare of the laboratory mouse. Much of this information has yet to be translated into practice for environmental management of laboratory mice. In particular, the intricate and subtle physiological mechanisms by which mice sense and perceive their environment are generally ignored in specifications for - and provision of - the 'optimal' environment, potentially compromising health and welfare and the scientific validity of the research. Provision of a suitable micro-environment in the home cage is a prerequisite for good laboratory science and specifications for cage ventilation rate are mainly determined by the need to keep ammonia concentrations to an acceptable level.

Laboratory mice are routinely housed in one of three types of caging system; 1) open top wire-bar lid cages, 2) filter top cages and 3) actively (individually) ventilated cages (IVC). These three systems differ significantly in the ventilation rate of each cage and thus in the quality of the microenvironment within the cage. The atmospheric environment differs significantly between cage systems and depends on bedding type, cage cleaning frequency and ventilation rate (Reeb et al. 1998, Reeb-Whitaker et al., 2001). Cage cleaning requirements present a conflict between hygiene and disruption of scent-marking patterns (Olsson et al 2003) and associated pollutants, especially ammonia. Ammonia exposure may compromise olfactory perception by desensitising olfactory receptors with adverse consequences for reproduction (Everleigh, 1993), health (Gaafar et al 1992) and potentially behaviour.

Ammonia concentration in cages is often used as an indicator of the frequency with which cages should have their bedding changed to provide a refreshed environment for the mice. Many negative effects of high NH_3 concentration in the cage have been described. Concentrations of NH_3 from 25 to 250 ppm increased the severity of respiratory mycoplasmosis in rats during 4 to 8 weeks exposure (Broderson et al., 1976), and at 100 ppm for 1 to 4 weeks (Schoeb et al., 1982). The morphology of rat tracheal epithelium changed after only 4 days of exposure to 200 ppm NH_3 while the delayed type immune response was reduced in guinea pigs exposed to 90 ppm NH_3 (Targowski et al., 1984). Most recently, Bernard et al (2000) have suggested that elevated ammonia levels in the cage may impair embryo production in superovulated mice.

The human occupational exposure limit (OEL) for NH_3 exposure during an 8-hour working day of 25 ppm (ACGIH, 2000) is normally taken as the tolerable concentration for rodents. The first signs of effect in rodents, such as lower resistance to pathogenic organisms, can be seen at about this concentration. However, no studies have been performed to determine the tolerance of rodents to atmospheric ammonia. The past trend towards filter-top cages may therefore have provided an aversive environment for mice unless the bedding was changed at least every four days, for example. Likewise the current trend towards IVCs may alleviate this situation, but bedding management in these systems needs to be based upon an evaluation of the animals' perception of their atmospheric environment, in particular the concentration of ammonia.

The research presented in this paper builds upon substantial evidence that pigs and chickens have a significant preference for fresh air over ammoniated atmospheres, at concentrations that occur in many livestock buildings (Jones et al., 1996; Kristensen et al., 2000; Jones et al., 2005). The objectives of this project were to determine the behavioral responses of laboratory mice to

ammoniated atmospheres at concentrations commonly found in laboratory animal facilities, specifically:

1. to design, construct and calibrate an environmental preference chamber for laboratory mice;

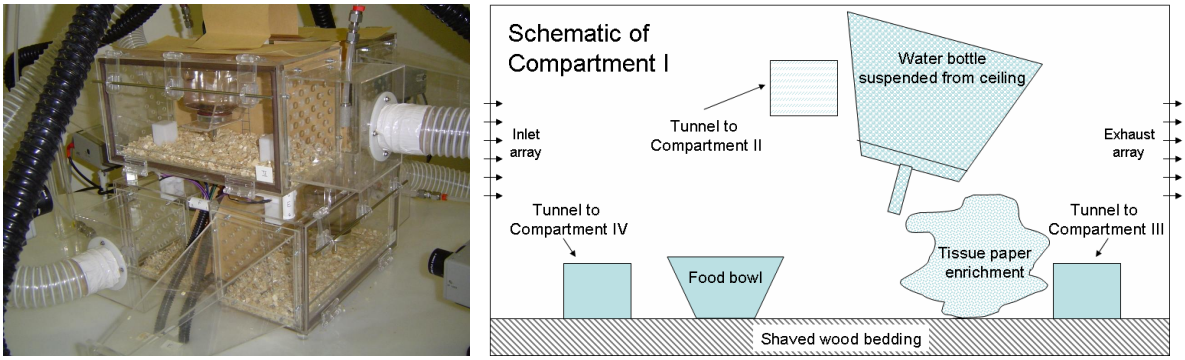
2. to carry out a pilot test of the chamber with mice; and

3. to determine the behavioral responses of mice to atmospheric ammonia in an experiment.

Materials and Methods

Preference Chamber Design

An environmental preference chamber was designed and built (Figure 1). It comprises four connected compartments (each 300 x 150 x 150 mm), arranged on two tiers, each of two compartments. Mice can move between the compartments via ladders and short access tubes. This design is preferable to the more conventional design of four compartments arranged in a cross on one level because the short access tubes do not provide an area for mice to dwell or tarry and the three-dimensional design with ladders will encourage climbing activity (Nevison et al., 1999). Thus a mouse can access all the alternative compartments from any one compartment.



Figures 1 and 2. Environmental preference chamber for laboratory mice (left) and schematic of Compartment I within the chamber (right).

The chamber was built from clear Perspex to allow the mice to be observed with mini-video cameras, mounted outside the chamber. Image quality and resolution of mouse behavior were determined in a preliminary trial. Special attention was paid to the compartments' illumination to ensure a uniform intensity, i.e. 0-5 lux with room lights off (the tracking system generated a small amount of light near the tunnels) and 25-50 lux with room lights on and distribution of light similar within each compartment (brighter at each end). The back wall and access tubes of each compartment were covered with a paper of color similar to the bedding to create contrast for the cameras and to block some of the light from the room.

Each compartment was ventilated separately at ≈100 air changes per hour to minimize pollutant build up. Air velocity was maintained below 0.1 ms^{-1} by the use of an array of inlets (41 circular openings each of 7 mm diameter) in the end wall. The inlets and outlets were designed to promote a uniform laminar flow through each compartment. The outlet area was sufficient to minimize effect on flow and resulting back pressure in each compartment (41 circular openings each of 8mm diameter). Cross-flow between the compartments was not desired; however, it was expected that ventilating each compartment equally would result in equal static pressures across the tunnels and minimal air

would pass from one compartment to another. Therefore, no covering was used over the tunnel openings.

The tunnels were instrumented with infrared sensors and photosensors for tracking movement through the tunnels. An infrared sensor pair was placed at each end of each tunnel; one photosensor was placed in the center of each tunnel. A white LED was located next to the photosensor in the center of the tunnel to provide consistent light in light and dark conditions. All sensors were scanned continuously. Data were written to file for any instance in which the beam between any IR sensor pair was broken. The resulting data were a series of 0's and 1's for every sensor pair to indicate 'not in tunnel' or 'in tunnel' respectively. The photosensor returned a resistance value, which varied according to the reflectance of the object in front of it.

Each compartment in the chamber was furnished (Figure 2) with 85g bedding material (softwood shavings, approximately 1.5cm), a piece of 2-ply tissue paper (approximately 15cm x 30cm), a hanging water bottle, and 30g of pellet food. The entire chamber and components were cleaned and disinfected (by wiping the chamber and briefly soaking the water and food containers with Virkon S) between mouse batches.

Preliminary Chamber Testing

Ventilation

The physical performance of the chamber was tested prior to use with animals. To test the cross-flow of air between compartments, ammonia was injected into each compartment and the exhaust air from all compartments was monitored for ammonia concentration. In only one compartment was there a shift of approximately 3ppm in exhaust ammonia concentration, which represented a minimal and acceptable amount of inter-compartment leakage. A PC-controlled, ammonia gas analyzer was used to monitor continually ammonia concentrations throughout the experiment in each compartment and to regulate the rate of supply of ammonia using mass flow controllers. Temperature and relative humidity were also recorded with a data logger. Daily verification of ammonia concentration was completed using diffusion tubes (Draeger) placed in the exhaust pipe of each compartment.

Mouse Usage of the Preference Chamber

Once the physical performance of the preference chamber was verified, pilot testing was carried out to determine the usage of the chamber by the mice, particularly the tunnels. Several methods of training the mice to navigate the tunnels were explored. Exploration of different compartments occurred sooner if mice were placed in separate compartments initially instead of together in the same compartment. The most appropriate breed and size of mouse was also determined in the pilot test, i.e Balb/c, 21-27 days old, 15-19g. Additionally, different methods for coloring the fur were tested for individual identification via the photosensor of the tracking system. Permanent marker, food coloring, watercolor, pig marker, and sheep marker were all tested for color intensity and consistency over several days (both of which were required for useful output from the photosensor). The most feasible method was the pig marker, though the consistency over several days was sub-optimal.

Experimental Design

Husbandry

The husbandry of the mice followed normal practices. Holding accommodation was provided by a ventilated cage rack, supplied by BioZone Inc. This ensured that the animals were kept in fresh air

prior to the preference test. Temperature and humidity control were provided by the room's system. Mice were provided a light cycle of 12 hours light/12 hours dark. Food and water were available *ad libitum* in both the holding rack and the preference chamber. Normal contact bedding material (softwood shavings) were used and changed weekly in the holding rack. A sheet of 2-ply tissue paper was provided in both the holding rack and the preference chamber for enrichment. All components in the holding rack were disinfected between groups of mice with Virkon S.

On arrival, the mice were randomly separated into groups of four mice and placed in prepared cages in the holding rack. Mice remained in the holding rack for at least 7 days of acclimation prior to being placed in the preference chamber. Each mouse was picked up at least once each day during the acclimation period to also acclimate to human handling.

Experiment Protocol

The objective of the main experiment was to assess the behavioral responses of laboratory mice to atmospheric ammonia in a preference test. The experimental design was a balanced factorial design based on Latin squares with ammonia concentration and compartment as the experimental factors with four levels each (nominal concentrations of 0, 25, 50 and 100 ppm, and compartments I,II, III and IV respectively). There are 24 sets of arrangements of ammonia concentration in the four compartments of the chamber but only 4 were tested since this number was sufficient to give a powerful experiment with a minimal number of experimental animals. The experimental unit is the compartment and not the individual mouse or group of mice.

Thus 4 groups, each of four female (Balb/c, 21-27 days old, 15-19g) mice were given a preference test lasting four days. Time lapse video recordings were used to verify the results obtained by the tracking system.

The first two days of each trial comprised an acclimation period for the mice to the chamber. Each mouse was placed in a separate compartment within the chamber. For the first 24 hours, the mice were allowed to explore the chamber with no interference. After 24 hours, the movements and locations of each mouse were reviewed. For mice that had not navigated all tunnels both directions, additional training was necessary. Training consisted of holding each mouse closely in front of a tunnel through which it should pass. This was sufficient motivation for all mice to pass through the tunnels. The mice were then allowed a minimum of 30 minutes of exploration before repeating the training, if needed. This was continued until an evident loss of explorative behavior was observed. If needed, the process was repeated after at least 3 hours had passed.

The following rules were used to evaluate whether or not a trial would continue for the final two days with ammonia treatments.

Rule 1: Each mouse must be familiar with each of the four compartments, each of which had similar resources.

Rule 2: Each mouse must be aware that it is a member of a group and is free to interact socially or abstain from joining the group (unless the group comes to it).

Rule 3: Each mouse must demonstrate the ability to move independently through both a horizontal and a vertical tunnel (with no human intervention).

After 48 hours, the mice were removed from the chamber and placed in their holding cage in the holding rack for approximately 30 minutes. The bedding, food, and tissue in the preference chamber were replaced with fresh furnishings; however the chamber was not disinfected. This procedure ensured that equal resources were available in all compartments when the ammonia treatment was applied.

The mice were then returned to the chamber, each in separate compartments, and allowed one hour of re-familiarization and exploration prior to the application of treatment. For the final 48 hours, the treatment was applied. Ammonia was injected via a mixing plenum into the air stream of each compartment at a controlled flow rate to maintain ammonia concentrations of nominally 0, 25, 50 and 100 ppm. The compartments were not opened or accessed during the final 48 hours of the experiment.

The liveweight of each mouse was also measured before and after the preference test. The experiment was authorized under the Animals (Scientific Procedures) Act 1986 (UK) after ethical review. At the end of the trial, the mice were killed humanely using a Schedule 1 method.

Data Analysis

The data collected with the tracking system were processed to give a set of mouse movements and corresponding times, using the following steps.

1) Identify presence of a mouse in a tunnel
2) Identify direction of movement through the tunnel
3) Summarize list of movement into and out of each compartment
4) Organize data into four sets, one for each compartment (moves into and out of)
5) Calculate amount of time spent in each compartment (time into less time out of)
6) Calculate the number of mice in each compartment at a given time
7) Calculate the variables for statistical analysis
    a. total mouse hours in compartment
    b. percent of total time in compartment
    c. number of moves into each compartment
    d. average duration of stay in compartment
    e. mean number of mice in a compartment at a given time
    f. mean hours spent in each compartment per mouse

The video images were watched to verify the recordings of the tracker, specifically a check-off for identifying direction of moves and time of move, as well as the number of mice in a compartment at a given time. Adjustments were made as needed, specifically for moves unidentifiable by the algorithms.

Summaries were completed for the entire 48 hours, 0-24 hours, 24-48 hours, and periods when the lights were on and off.

The data set for statistical analysis consisted of the six variables listed above. Each set was analyzed separately in SAS with the PROC MIXED procedure with model effects of ammonia treatment, location of the compartment, and trial. Effects were considered significant at $\alpha=0.05$.

Results

Mouse Behavior

With the training procedures described above, all mice learned to navigate independently the chamber. During the first 48 hours of familiarization, each mouse was trained and observed to move

independently across a horizontal tunnel and both up and down a vertical tunnel, which satisfied the stated rules. After learning to navigate the chamber, the mice moved frequently between compartments. The total number of moves of all mice between compartments in 48 hours (with ammonia present) was: 3309, 2221, 2046, and 2470, for trials 1, 2, 3, and 4, respectively.

The longest periods of time spent in the same compartments were during sleeping, usually in one of the two compartments on the top tier, though not all groups preferred the same compartment. The mice ate and drank in all compartments. They also handled nesting material in all compartments; however, the most organized nests were in the preferred sleeping compartments. In two trials, the mice moved nesting material through the tunnels into the preferred sleeping compartment (in both cases, the top tier compartment with the highest concentration of ammonia), with the mice in one trial moving all nest material in the entire chamber into the same compartment.

From the statistical analyses, the results were similar for each breakdown (overall, lights on, lights off, 0-24h, 24-48h). The effect of compartment location was significant for most variables, and the effect of trial was significant for the number of moves for the first 24 h. The effect of ammonia treatment was not significant for any variable in any analysis. Compartments I and II (on the top tier) tended to be different from III and IV (bottom tier), but not from one another. For the final 24 hours, compartments I and II were different from one another, with compartment I being preferred regardless of ammonia concentration.

For the first 24 hours in the ammoniated compartments, preferences were less defined (Table 1) and only the effect of compartment location ($P=0.05$) and trial ($P=0.01$) were significant in the model for the number of moves. However, for the final 24 hours in the ammoniated compartments, clear preferences were observed (Table 2). All variables were highly significant for compartment location ($P<0.001$). The means for each variable demonstrate the strong preference for the top tier of the chamber over the bottom tier, but no distinguishable preference for or aversion to different concentrations of ammonia (Table 3).

Table 1. Statistical results (P-Value) for mouse time allocation during 0-24 h

Variable/P-Value	Ammonia Treatment	Compartment Location	Trial
Total mouse hours	0.38	0.13	1.00
% time in compartment	0.39	0.13	1.00
Number moves into	0.81	0.05	0.01
Mean stay duration, min	0.26	0.21	0.85
Mean # mice/comp.	0.39	0.13	1.00
Mean total hours/mouse	0.37	0.12	1.00

Table 2. Statistical results (P-Value) for mouse time allocation during 24-48 h

Variable/P-Value	Ammonia Treatment	Compartment Location	Trial
Total mouse*hours	0.19	0.0002	0.99
% time in compartment	0.17	0.0001	1.00
Number moves into	0.61	0.0006	0.07

Mean stay duration, min	0.29	0.001	0.90
Mean # mice/comp.	0.16	0.0001	1.00
Mean hours/mouse	0.18	0.0002	0.99

Table 3. Mean by treatment, compartment, and trial for 24-48 h for variables

Variable/Mean (+/-SE)	Ammonia Treatment, ppm NH_3			
	0	25	50	100
Total mouse hours	27.4(12.8)	21.9(11.3)	28.1(14.7)	16.1(9.0)
% time in compartment	29.6(14.2)	23.0(11.8)	29.6(15.4)	17.8(10.1)
Number moves into	337(51)	332(37)	310(56)	305(80)
Mean stay duration, min	4.5(1.9)	3.6(1.6)	4.7(2.0)	2.9(1.0)
Mean # mice/comp.	1.2(0.6)	0.9(0.5)	1.2(0.6)	0.7(0.4)
Mean total hours/mouse	6.9(3.2)	5.5(2.8)	7.1(3.7)	4.2(2.4)

Variable/Mean (+/-SE)	Compartment Location			
	I	II	III	IV
Total mouse hours	57.4(5.7)[a]	21.8(5.2)[b]	7.4(0.6)[c]	7.0(0.8)[c]
% time in compartment	61.8(5.7)[a]	23.0(5.3)[b]	7.8(0.5)[c]	7.4(0.7)[c]
Number moves into	412(22)[a]	409(22)[a]	258(28)[b]	205(29)[c]
Mean stay duration, min	8.7(1.1)[a]	3.2(0.6)[b]	1.8(0.2)[b]	2.1(0.1)[b]
Mean # mice/comp.	2.5(0.2)[a]	0.9(0.2)[b]	0.3(0.02)[c]	0.3(0.03)[c]
Mean total hours/mouse	14.6(1.3)[a]	5.5(1.3)[b]	1.9(0.2)[c]	1.8(0.2)[c]

Variable/Mean (+/-SE)	Trial			
	1	2	3	4
Total mouse hours	23.2(8.7)	24.0(11.6)	22.6(13.2)	23.8(15.3)
% time in compartment	25.0(9.7)	25.0(12.1)	25.0(14.6)	25.0(16.1)
Number moves into	357(65)[a]	299(66)[b]	276(51)[b]	352(31)[b]
Mean stay duration, min	3.6(0.9)	4.1(1.4)	4.2(2.0)	3.8(2.3)
Mean # mice/comp.	1.0(0.4)	1.0(0.5)	1.0(0.6)	1.0(0.6)
Mean total hours/mouse	6.0(2.3)	6.0(2.9)	5.7(3.3)	6.0(3.8)

[a, b, c] denotes statistically different for given variable ($P<0.1$)

Tracking system

In general, the performance of the tracking system was highly satisfactory but a few inaccuracies were apparent with the analysis as described. Specifically, the calculation of the number of mice in a compartment at a given time: when that number exceeded the possible values of 0-4, an adjustment was necessary and any discrepancies were eliminated by the back-up video observations.

All moves identified by the IR sensor pairs were correct. There were a large number of unidentified moves that were clarified by watching the video. There were several moves identified incorrectly as non-moves, which were also clarified by the video observations. In a very few instances, a move was not identified by the tracking system, usually because one mouse closely followed another through a tunnel, with no clear separation.

The performance of the identification system was poor and it was unable to identify individually the colors on the fur of each mouse. The pig marker used was not consistent in intensity such that a statistically significant difference between each color could be discriminated.

Discussion

This experiment is the first study to determine the tolerance of laboratory mice for atmospheric ammonia at concentrations that are typically found in laboratory animal facilities. The development of an environmental preference chamber clearly has many other applications than the one used here and there are further refinements that could be made to improve the performance of the chamber and its associated tracking system.

We believe that the design of the chamber is particularly novel since access to all the available choices is possible from any one compartment, which overcomes a significant limitation of the alternative designs, e.g. a radial maze or an annulus, where either animals have to traverse a neutral central compartment with difficulties in interpreting time spent in the centre, or access to alternative choices is limited to those in the neighboring compartments and animals have to traverse these to access more distant choices, respectively. However, while the mice obviously made full use of the chamber – and may have valued the spatial enrichment provided - we need to determine the reason why they showed a clear preference for the upper tier of compartments, which may have been due to an unknown environmental heterogeneity or simply an attraction for height. Whatever the reason, the design of the experiment catered for such a possibility.

Other improvements could be made to the apparatus. The tracking system was more efficient in recording mouse movements and time spent in each compartment than the video observations but did not provide information on specific behaviors. It significantly reduced the amount of time required to watch the video tapes. The photosensor for individual identification did not give accurate information because of the behavior of the mice, which meant that the preferences of individual mice could not be determined. Grooming changed the intensity of the color applied, and we are not aware of a superior dye. Further refinements to the tracking system could be made, perhaps based upon implanted radio-transmitting tags.

Environmental preference tests are widely used in studies of animal welfare and their basis is simple: animals are given a choice between several environments and observations are made of the relative usage. An animal's choices are normally assumed to reflect its behavioral needs and to be in the best interests of its welfare. The limitations of the test are well recognized (Duncan, 1992) and, in the context of this research, are twofold. Firstly, preferences may be affected by prior experience; our mice were reared in fresh air with little prior experience of chronic exposure to atmospheric ammonia. Secondly, preferences reflect relative choices. This can be overcome by providing a wide range of (four) atmospheres and including fresh air as an absolute base line.

The results showed that there were two motivations acting in mouse behaviour. The mice clearly preferred to be in the two compartments on the top tier of the chamber rather than in the bottom. This may be because the greater height offered them a better view or an experimental artefact of an unknown environmental heterogeneity. They explored the other compartments and regularly patrolled their environment.

Surprisingly, the mice did not exhibit a clear preference for or aversion to ammonia concentration. Once familiarization had finished and the ammonia treatment had commenced, they still entered and explored every compartment, even those that had ammonia concentrations as high as 100 ppm. This apparent lack of aversion continued for the second period of 24 hours despite a strong preference for the top tier compartments, which included the higher ammonia concentrations in some

trials. Thus a strong compartment preference was not overcome by any preference for fresh air over an ammoniated atmosphere.

In similar experiments, pigs and chickens (Jones et al., 1996; Kristensen et al., 2000; Jones et al., 2005) showed a strong but delayed aversion to ammonia concentrations of 20 ppm and higher. The reasons why mice did not show a similar preference may be related to their reliance on olfaction: olfactory cues are particularly important for sexual, social and maternal behaviour. Ammonia may be associated with urinary odors employed in territorial scent marking and the mice may have been 'confused' by the elevated concentrations which they encountered. Whatever the explanation, the mice did not make a short-term choice that was in their long-term interest.

The results of this experiment have implications for the design and management of laboratory animal facilities. Pending confirmation of these results, existing guidelines for the acceptable concentration of ammonia should be retained since the apparent tolerance by mice of ammonia could be interpreted as a license to permit potentially noxious concentrations of ammonia to be allowed. This would harm health, reproduction and normal behavior.

This project provides an entry into future research on the environmental preferences and motivations of laboratory rodents. The applications are manifold and include other gases, lighting, temperature, and social factors such as group size. The ultimate goal would be to provide a physical environment for laboratory animals that accords with their preferences, meets the highest standards of care and does not interfere with the validity of scientific tests.

Conclusion

A novel preference chamber with four compartments was designed and built to test the aversiveness of atmospheric ammonia to laboratory mice. An experiment was successfully undertaken with four batches, each of four mice, which were given the choice between nominal ammonia concentrations of 0, 25, 50 and 100 ppm over two days. The results showed that there were two motivations acting on mouse behavior. The mice preferred the two compartments of the top tier of the chamber to those on the lower tier. They explored the other compartments and regularly patrolled thechamber. The mice did not exhibit a clear preference for or aversion to ammonia, which implies that their short-term tolerance of ammonia at potentially noxious concentrations may not be in their long-term interest.

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