Comparative Medicine
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Vol 57, No 4
August 2007
Pages 355–359

Effects of Cage Density on Behavior in Young Adult Mice

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Optimal housing conditions for mice can be achieved by minimizing environmental variables, such as those that may contribute to anxiety-like behavior. This study evaluated the effects of cage size on juvenile mice through assessment of differences in weaning weight, locomotor skills, and anxiety-like behavior. Eighteen pairs of male and pregnant female Swiss–Webster (Cr:SW) mice were housed in 3 different caging scenarios, providing 429, 505, or 729 cm^2 of space. Litters were standardized to 10 pups per litter in each cage. Mice reared in each caging scenario were assessed with the open-field, light–dark exploration, and elevated plus-maze tests. No differences in weaning weight were noted. Mice reared in the 505- and 729-cm^2 cages explored a significantly larger area of the open-field arena than did those in the 429-cm^2 cages. Those reared in the 505-cm^2 cages spent more time in the center of the open field than did those in the 729-cm^2 cages, suggesting that anxiety-like behavior may be increased in the animals housed in the larger cages. This study did not establish a consistent link between decreased floor space and increased anxiety-like behavior; neither does there appear to be a consistent effect of available floor area on the development of locomotor skills on mouse pups.

Abbreviations: EPM, elevated-plus maze; LD, light–dark exploration test; OF, open-field exploration test

Optimal housing conditions for mice can be achieved by minimizing environmental variables that may contribute to anxiety-like behavior. The number of animals housed per cage potentially can alter research variables, including behavioral and physical parameters, thereby affecting the development of appropriate research models. The *Guide for the Care and Use of Laboratory Animals*[20] offers recommendations on the amount of cage floor space for group-housed laboratory mice of various weights. These values have been established on the basis of professional judgment and experience. Current literature assessing murine cage density and its effect on mice includes many studies that evaluate the effects of cage density on environmental conditions and interaction of conspecifics.[12,16,22,24,26] Smith and colleagues[23] reported that C57BL/6J mice as large as 29 g may be housed with 36.13 cm^2 of floor area per mouse, half of the space recommended by the *Guide*,[20] without significantly affecting weight gain, food consumption, urinary testosterone levels, incage carbon dioxide concentrations, or temperature. McGlone and colleagues[18] concluded that 32.2 cm^2 per mouse did not cause behavioral, health, immune, or performance problems for BALB/cJ mice. However, a paucity of literature addresses the potential effects of rearing cage density on anxiety-like behavior in juvenile mice.

Anxiety-like behavior has been described in mice based on the performance and assessment of animal models of human anxiety.[3] Being anxious is an adaptive response to an unfamiliar environment, especially when confronted with danger or threat.[21] Anxiety-like behavior is thought to result from the conflict-inherent approach–avoidance situation.[6] Cage density, independent of group size, is a parameter that might influence anxiety-like behavior in mice. Open-field testing is based on the belief that anxious mice will stay in the periphery of the test apparatus and exhibit increased defecation and grooming behaviors.[3] Anxiety-like behavior resulting from housing conditions has been evaluated in relation to the availability of anxiolytics and different caging scenarios, including the use of enrichment devices.[1] However, the link between cage density and the development of anxiety-like behavior in mice has not been proven conclusively.

Cage density and its potential effects on mouse health and behavior are important to the research community because these factors could directly affect the scientific validity and reproducibility of the data.[27] Previous studies indicate that cage density can play a role in murine health,[8,10,19,24] but only a limited number of published studies are available. We designed the present study to contribute to the body of knowledge in this area.

Harmonization of animal care and use, including housing parameters, is considered to be beneficial in the areas of toxicology and regulatory testing in both Europe and the United States.[25] The need for data-driven housing guidelines affects all aspects of biomedical research. Attaining global harmonization of mouse-housing parameters has been promoted in the laboratory animal community, and assessing the effects of rearing cage density on young adult mice can contribute to the body of data that directs this movement.[28] This goal is an important one and requires a team effort between the scientist, veterinarian, institutional animal care and use committee, and animal care staff, with the first step in harmonization possibly being that of harmonization of study protocol.[27]

The study objective was to evaluate differences in weight, locomotor skills, and anxiety-like behavior among young adult mice reared in 3 different-sized cages.

Received: 2 Nov 2006. Revision requested: 3 Dec 2006. Accepted: 20 Mar 2007.
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Materials and Methods

To compare effects of housing density, this study used 3 cage sizes that represent standard mouse caging available in our facility (Table 1). Group size for each cage was set at 2 adults and 10 pups. The recommended space allowances for group housed laboratory rodents in the *Guide*[20] were referenced when calculating the appropriate amount of floor space to provide 2 adult mice weighing greater than 25 g and 10 pups weighing less than 15 g each. The group C cage size approximated the space recommended in the *Guide*[20] and was the reference cage. Group A had 60% of the floor space of group C, and Group B had 69% of the floor space of group C.

Behavioral tests, such as the open-field exploration (OF), light–dark (LD), and elevated-plus maze (EPM) tests, are all based on the innate aversion of rodents to open, brightly illuminated areas.[3,13,17] We used the OF, LD, and EPM tests to assess locomotor ability and anxiety-like behavior of each mouse. Locomotor abilities were verified by the OF test, which in turn ensured appropriate ambulatory ability for both the LD and EPM tests. Mouse movement between the light and dark compartments of the LD test is used as an index of activity or exploration. Mouse habituation over time and the time spent in each compartment is a reflection of aversion.[13] The EPM test improves on the LD test by adding new components, height and the openness of the arms, which are raised a meter above the floor. The EPM is often used to test anxiolytic drugs, and the total number of entries into all arms provides a built-in control measure for general hyperactivity or sedation.[6]

Subjects were tested once on each behavioral apparatus.[9] According to Bessa and colleagues, previous exposure to the EPM test is recognized as an important modifier of performance, with retesting of rodents increasing open-arm avoidance.[2] Similarly, a phenomenon termed 'one-trial tolerance' has been reported in both the EPM and the LD tests. There is marked attenuation or even abolition of anxiolytic-like effect of benzodiazepines in mice by a single previous test experience.[11]

Mice. Cr:SW mice, an outbred strain that typically produces large litters, were chosen for the study to increase the likelihood of generating at least 10 pups per litter. We purchased 48 mice consisting of 24 males and 24 late-term pregnant females (Animal Production Area, National Cancer Institute, National Institutes of Health, Frederick, MD), which we received in 4 equivalent shipments of 6 male and 6 female mice. We used 18 pairs of mice for the main study, with 6 pairs used to produce additional pups to supplement litters that were smaller than the requisite 10 pups. To create 3 different cage density scenarios, litters were standardized to 10 pups each by either fostering within the first 1 to 2 d after birth or euthanizing excess pups. Pups and adults were housed for 21 d in cages of 1 of 3 different sizes, after which time each pup was sexed, ear-tagged with identifying numbers, and weighed to analyze for differences.

For each litter of 9 to 10 pups weaned, pups were separated into same-sex groups (to avoid pregnancy). Because the litters did not always have equal numbers of male and female mice, the same-sex groups consisted of 3 to 5 mice per 429-cm^2 'shoebox cage' (Techniplast USA, Exton, PA). Therefore, postweaning conditions were standardized by either meeting or exceeding *Guide* recommendations for available floor space. Study animals were housed in these cages from the time of weaning, throughout behavioral testing, until the end of the study.

Mice were kept in a specific pathogen-free animal care facility where quarterly sentinel surveillance was done. Sentinel mice were negative for *Mycoplasma pulmonis*, Sendai virus, mouse hepatitis virus, pneumonia virus of mice, reovirus 3, Theiler virus, ectromelia, mouse adenovirus, polyoma virus, lymphocytic choriomeningitis virus, cytomegalovirus, murine rotavirus, murine parvovirus, cilia-associated respiratory bacillus, and *Salmonella* spp. Mice were provided feed (NIH-31 Autoclavable Rodent Diet, Ziegler Brothers, Gardner, PA) and water ad libitum. Corncob bedding (The Andersons, Maumee, OH) and nestlets (Ancare, Bellmore, NY) were placed in each cage. Cages were handled and changed by using aseptic microisolator technique for microbiologic control. Complete cage changes were done twice-weekly. The light cycle was 12:12-h light:dark. Euthanasia was accomplished with CO_2 gas, or mice were transferred to other approved protocols within the institute. This study was reviewed and approved by the institutional animal care and use committee and conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Table 1. Group definitions, caging measurements, and number of pups evaluated in each group

Group	Total floor area (length × width × height)	No. of pups
A	429 cm^2 (33 × 13 × 12 cm)[a,c]	59[d]
B	505 cm^2 (27 × 18.7 × 15.3 cm)[b,c]	60
C	729 cm^2 (27 × 27 × 12.7 cm)[b,c]	58[e]

[a]Techniplast USA, Exton, PA.
[b]Thoren Caging Systems, Hazleton, PA.
[c]Internal cage dimensions adjusted for bedding placed in each cage.
[d]One pup from the group A died during ear tagging, but housing density remained greater than *Guide* recommendation.
[e]2 litters in group C each consisted of 9 pups, but housing density remained less than *Guide* recommendations. Fostering was not possible for these litters because of timing of foster litter.

Cages. Male–female pairs and offspring were housed in 3 different-sized polycarbonate, static filter-top cages of 429 (group A), 505 (group B), and 729 cm^2 (group C; Table 1). All cage components that were in direct or indirect contact with the mice (including feed, bedding, and water) were autoclaved or otherwise suitably disinfected.

OF test. At 7 wk of age, mice were assessed by using an automated OF system.[4] Time spent in the center of the open-field arena and total distances explored were recorded by an automated tracking system (Ethovision, Noldus Information Technology, Leesburg, VA). Mice were acclimated to the testing room with white noise (Sound Screen 980, Juno Beach, FL) for 1 h before all behavioral tests were performed. Each subject was placed in 1 corner of the testing arena and then released and allowed to explore the arena for 10 min. The open field was a square arena (40 × 40 × 35 cm) with clear acrylic walls and floor. Three arenas were arranged adjacent to each other for simultaneous recording of 3 test animals. Between subjects, each arena was cleaned with 70% ethanol.

LD test. At 8 wk of age, mice were assessed using the LD test.[14] The test apparatus (Med Associates, St Albans, VT) consisted of 2 distinct compartments of equal size separated by an open guillotine-style door constructed from polycarbonate material. The overall inside dimensions were 46.5 × 12.7 × 12.7 cm. Each compartment measured 23.3 cm in length. One compartment was all black, the other all white. Each compartment had a hinged, clear, polycarbonate lid with a built-in light fixture. The white compart-

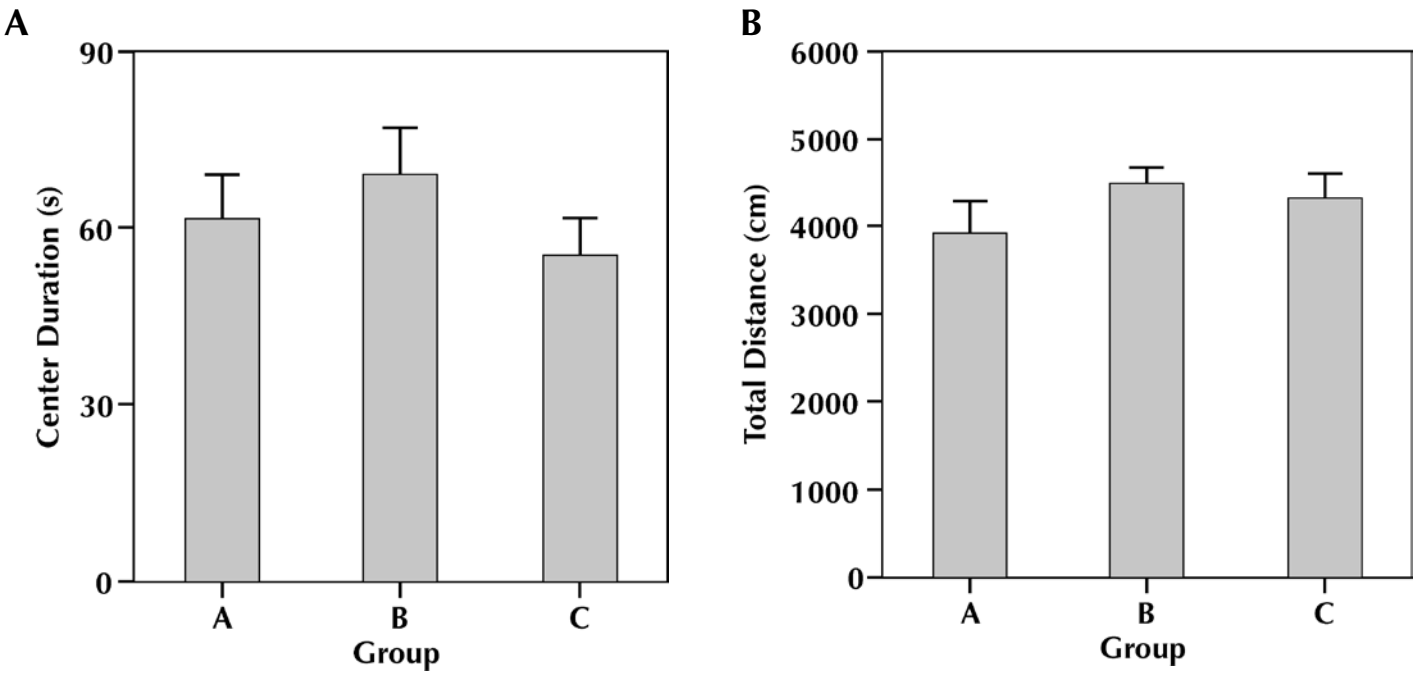

Figure 1. Open-field exploration. (A) The total amount of time spent in the center of the open field and (B) the total distance traveled over 10 min are shown for mice in each cage size. Data are presented as means, with error bars denoting the 95% confidence interval of the mean.

ment was illuminated during testing, and the black compartment was not. The testing chamber was enclosed in a sound-attenuating cubicle measuring 66 × 55.9 × 35.6 cm (Med Associates).

Each mouse was placed in the dark compartment, facing away from the partition door, and allowed to explore both sides of the apparatus for 10 min while the number of light–dark transitions between the 2 compartments and the total time spent in the dark compartment were scored by using place-preference data collection software (Med Associates). The apparatus was cleaned with 70% ethanol between subjects.

EPM test. At 9 wk of age, mice were assessed using the EPM test.[17] Two closed arms (66 × 5 × 15 cm) and 2 open arms (66 × 5 cm) comprised the apparatus (San Diego Instruments, San Diego, CA) that extended from a common central platform. A small raised lip (0.3 cm) around the perimeter of the open arms prevented the mouse from falling. The maze was constructed from acrylonitrile-butadiene-styrene plastic, with black floor and walls, and stood 50 cm above floor level.

Mice were placed individually in the center area, facing an open arm and allowed to explore the field for 5 min. Open- and closed-arm entries were recorded by using the video tracking system (Ethovision, Noldus Information Technology). Behaviors scored were open and closed arm entries (an arm entry was defined as all 4 paws in an arm) and time spent in the open arms. The apparatus was cleaned with 70% ethanol after each subject.

Statistical analysis. We used 1-way analysis of variance to compare the 3 rearing-cage densities studied (groups A, B and C) regarding weaning weight, locomotor skills and anxiety-like behavior. The Student–Newman–Keuls multiple comparisons procedure was used to perform pairwise comparison of the 3 rearing cage densities after obtaining a statistically significant F-test by 1-way analysis of variance. All analyses were performed by using the SPSS 12.0 (SPSS, Chicago, IL) software package. The threshold for statistical significance was set at $P = 0.05$ for all tests.

Results

Mean weaning weight did not differ among groups A, B, and C ($F_{2, 174} = 0.841$, $P = 0.433$). In the OF test, the time (s) spent in the center of the field and the distance traveled in the arena (cm) were analyzed. Statistically significant differences among the 3 rearing-cage densities were obtained for duration in the center of the field ($F_{2, 174} = 3.531$, $P = 0.031$; Figure 1 A) and total distance explored ($F_{2,174} = 4.105$, $P = 0.018$; Figure 1 B). Pairwise comparison of the 3 rearing cage densities revealed that mice in group B spent significantly more time in the center of the field than did those in group C, indicating a decreased avoidance of the open area. However, duration in the center of the open field did not differ between groups A and C or A and B. In addition, mice in group B explored a significantly ($F_{2,174} = 4.017$, $P = 0.020$) larger area of the field than did those in group A, but there were no significant differences between groups A and C or B and C. These findings can be interpreted as decreased anxiety-like behavior in group B (Table 2).

Significant effects of rearing-cage size were not detected in the LD test for the number of light–dark transitions ($F_{2, 174} = 1.934$, $P = 0.148$) or total time spent in the dark compartment ($F_{2, 174} = 0.319$, $P = 0.727$) of the LD test or in the EPM test for total time spent in the open arms ($F_{2, 174} = 1.006$, $P = 0.368$), the number of open-arm entries ($F_{2, 174} = 1.173$, $P = .312$), or the number of closed-arm entries ($F_{2, 174} = 0.068$, $P = 0.935$).

Discussion

Weaning weight at 3 wk of age did not differ among the mice at the 3 rearing-cage densities. The results from the present study are consistent with those from previous experiements[18] in adult mice, which have shown that body weight is unaffected by cage density.

The OF test is the most commonly used general measure of motor function.[6] Group A mice explored a smaller area of the open-field apparatus than did those in group B. The distance traveled

Table 2. Test parameter findings for Groups A, B and C

Test parameter	mean ± 1 standard deviation (minimum, maximum)		
	Group A	Group B	Group C
Weaning weight (g)	11.2 ± 1.1 (7.4, 14.2)	11.4 ± 1.7 (4.1, 14.8)	11.6 ± 1.0 (10.0, 14.3)
OF: center duration (s)[a]	61 ± 28 (16, 198)	69 ± 30 (23, 162)	56 ± 23 (2, 111)
OF: total distance traveled (cm)[a]	3929 ± 1345 (7.98, 6140)	4489 ± 760 (2722, 6331)	4323 ± 1106 (1130, 8797)
LD: no. of transitions	28 ± 7 (17, 53)	26 ± 6 (15, 46)	28 ± 7 (1, 47)
LD: time in dark compartment (s)	289 ± 44 (193, 424)	282 ± 50 (147, 396)	285 ± 57 (177, 572)
EPM: time spent in open arms (s)	66 ± 33 (3, 144)	75 ± 43 (5, 254)	67 ± 36 (6, 182)
EPM: no. of open-arm entries	14 ± 6 (4, 36)	13 ± 6 (2, 28)	13 ± 5 (3, 31)
EPM: no. of closed-arm entries	19 ± 5 (8, 34)	19 ± 6 (1, 39)	19 ± 5 (5, 33)

EPM, elevated-plus maze test; LD, light–dark exploration test; OF, open-field exploration test.
[a]$P < 0.05$ between 2 of the groups for this parameter.

by mice reared in Group C was not significantly different than those for those in group B. The group B mice traveled the longest distance in the OF test. According to the results of the present study, amount of floor area available has no dramatic effect on the development of locomotor skills in mouse pups.

Time in the center of the OF test assembly differed between groups B and C, but neither of these groups differed from group A. Although the OF test is not highly specific for evaluating anxiety-like behavior, the center duration time, a measure of time spent in the open area of the apparatus, provides some indication of this characteristic.[5] Mice in group C spent less time in the center of the field than did those in group B, indicating increased avoidance of the more threatening (that is, open) area of the apparatus by group C mice.[7] This difference can be interpreted as increased anxiety-like behavior in group C compared with group B mice. These results support previous reports that mice are less anxious and exhibited less aggressive behavior when reared in smaller enclosures compared with larger spaces.[18,26]

The LD test represents a natural conflict between the tendency of mice to explore a novel environment versus the tendency to avoid a brightly lit open field.[5] Similarly, the EPM test is based on the natural aversion of rodents for open spaces.[17] The LD test of exploration activity of Group A mice did not differ significantly compared with that of groups B and C. Given the results for total distance traveled during the OF test, we expected mice in group A to make fewer transitions between the light and dark compartments, but this was not the case.

The OF test is relatively insensitive as a measure of anxiety-like behavior, compared with the EPM and LD tests.[15] The lack of significant effects between groups in the LD and the EPM tests does not support findings with the OF test. Taken together, these results suggest that rearing cage size has a limited, if any, effect on anxiety levels in young adult mice.

Overall, data from the current study did not show a consistent effect of cage size on anxiety-like behavior in juvenile mice. In addition, the development of locomotor skills did not differ significantly among groups.

Acknowledgments

The authors thank Andrew Holmes and the animal care and technical staff of the National Institute on Alcohol Abuse and Alcoholism (NIAAA) for the use of behavioral test equipment and care of the experimental animals, respectively. We thank Patricia L Brown and Dale C Smith for constructive comments during manuscript preparation and Cara Olsen for invaluable assistance with statistical analysis. Financial support of this work was provided by the National Institutes of Health Office of Animal Care and Use and the NIAAA.

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