Journal of the American Association for Laboratory Animal Science
Copyright 2009
by the American Association for Laboratory Animal Science

Vol 48, No 6
November 2009
Pages 740–753

The Response of C57BL/6J and BALB/cJ Mice to Increased Housing Density

Anthony Nicholson,[1,*,†] Rachel D Malcolm,[1] Phillip L Russ,[1] Kristin Cough,[1] Chadi Touma,[2] Rupert Palme,[3] and Michael V Wiles[1]

Increased numbers of mice housed per cage (that is, increased housing density) is seen as 1 way to reduce the costs of conducting biomedical research. Current empirically derived guidelines are based on the area provided per mouse depending on body weight as documented in the *Guide for the Care and Use of Laboratory Animals*. The current study aimed to provide a more scientific basis for housing density by examining the response of C57BL/6J and BALB/cJ mice to increased housing density from weaning to 5 mo of age, to determine those parameters most useful for future larger-scale studies. A wide range of phenotypic characteristics—including growth rate, body composition, hematology, serum biochemistry, hormone and metabolite measurements, in-cage telemetry, behavior, and cage microenvironment—was examined at various time points. The parameters showing greatest changes were: growth rate, which was significantly reduced in animals at the highest density; adrenal gland size, the proportion of adrenal cortex, and concentration of fecal corticosterone metabolites, all of which were increased at higher densities; and anxiety and barbering, which were more pronounced at higher densities. Cage microenvironment deteriorated with increasing density, but the increases in measured parameters were small, and their biologic impact, if any, was not apparent. The current findings indicate that mouse housing density can be increased 50% to 100% above the current recommendations (as floor area per mouse) with no or few apparent affects on mouse overall wellbeing. However, weight gain, fecal corticosterone metabolite levels, and barbering differed significantly with housing density and therefore are suggested as good measures of the response to alterations in housing.

Abbreviations: CM, corticosterone metabolites; DEXA, dual-energy X-ray absorptiometry; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone.

Within the USA, space requirements for laboratory animals are outlined in the *Guide for the Care and Use of Laboratory Animals* (the *Guide*)[20] that states animals "must have enough space to turn around and to express normal postural adjustments … and must have enough clean bedding or unobstructed area to move and rest in." The *Guide* provides a sliding scale for the floor space requirements per mouse based on average body weight. The designated minimum floor space requirements (per mouse) are 6 in.^{2} (38.7 cm^2) for mice less than 10 g, 8 in.^{2} (51.6 cm^2) for mice 15 g or less, 12 in.^{2} (77.4 cm^2) for mice 25 g or less, and at least 15 in.^{2} (96.8 cm^2) for mice weighing more than 25 g. All cages must be at least 5 in. (12.7 cm) high, with no designated minimum cage size.[20] Furthermore, housing of mice in social groups rather than alone is encouraged whenever this practice is not in conflict with the study design or aims.

Previous studies at our institution demonstrated that housing mice of 4 commonly used inbred strains (C57BL/6J, BALB/cJ, NOD/LtJ, and FVB/NJ) for 8 wk in increasing densities up to a density twice that recommended by the *Guide* had no significant detrimental effects on the animals' grossly observable health or wellbeing as assessed by weight gain, food and water consumption, and urinary corticosterone.[45,46] Throughout the 8 wk of these studies, the cage microenvironment (air quality and temperature) was, with few exceptions, well within the limits suggested by the *Guide* and OSHA for environmental gas contaminants.

Studies using C57BL/6Crl and BALB/cJ mice housed from 3 to 6 wk of age for 5, 6, or 7 wk demonstrated limited negative effects of increased housing density.[13,30] Floor area per mouse tested in the cited 2 studies was 5, 10, 15, and 20 in.^{2} per mouse for mice housed in groups of 3. Mortality was lowest for those mice housed at the highest density, whereas plasma corticosterone levels, thought to reflect the level of stress experienced by animals, increased significantly with increased density. However, inhibitory effects on immune function, as might have been expected from increased corticosterone, were not detected.[11,40] Apart from increased plasma corticosterone, the studies did not detect indicators of reduced wellbeing even when mice were housed in one-third of the area recommended by the *Guide*, equivalent to a 3-fold increase in density. BALB/c was the strain most commonly used in earlier density studies, and because male BALB/c mice are known for their native aggression[23,53] their characteristic increased aggression was hypothesized to be a sensitive indicator of social stress associated with increased housing density. None of these previous studies concluded that increased housing density resulted in more aggressive behavior by male BALB/c mice.

These 2 groups of studies[13,30,45,46] highlight 1 of the many difficulties in performing experiments to assess housing density: the conundrum of distinguishing between floor area per mouse (cage size) versus group size. In 1 group of studies,[45,46] cage size was kept constant while group size was increased, whereas in the other studies,[13,30] group size was kept constant at 3 mice per cage while the area per mouse (cage size) was

Received:14 Jan 2009. Revision requested: 02 Mar 2009. Accepted:12 May 2009.
[1]The Jackson Laboratory, Bar Harbor, Maine; [2]Department of Behavioral Neuroendocrinology, Max Planck Institute of Psychiatry, Munich, Germany; [3]Biochemistry, Department of Natural Sciences, University of Veterinary Medicine, Vienna, Austria.
†Current address: Nicholson Anesthesia and Comparative Phenotyping Consultancy, Bar Harbor, Maine.
**Corresponding author. Email: annietone@roadrunner.com*

decreased. In practical terms these, studies had 2 different aims; the investigators for the first set of studies[45,46] were interested in using commercially available caging systems to assess the response of mice to increased group size as a way to increase housing density to try to determine the most suitable numbers of mice for a given cage type. In contrast, the researchers for the second group of studies[13,30] essentially had already decided on the 'ideal' group size and attempted to assess the response of mice to decreased cage area alone; this technique is useful to help determine which parameters might be best for assessing changes in cage density but is not entirely practical, nor economically feasible, in most vivaria given the standard cage sizes available and limited mouse room space. Interestingly, the 'ideal' group size determined by the second group of researchers[13,30] was similar to that resulting in the least aggression by male BALB/c mice.[52,53]

In the current study, we opted for the more practical, economic, and perhaps more realistic approach and varied group size by using cages that are standard at our institution. The previous studies[45,46] indicated that housing densities might be increased above the *Guide*'s recommendations with no, or few, strain-specific negative effects on the wellbeing of mice. However, we considered this conclusion to be limited by other contradictory data in many published studies (Figure 1). Further, direct comparison is confounded by variability in study design, including duration of housing, cage and group size, and the test parameters used to assess changes in animal wellbeing and welfare. In addition, few studies examined the effect of increased housing density over the prolonged periods of time often required for housing mice for colony maintenance and use in research projects.

In establishing the design of the current study, we had several aims. First, the current study is the first phase of a multiphase project designed to test a broad range of parameters and to focus on those tests for which there were large and statistically significant changes. Although the current sample size was small, we were particularly interested in identifying any robust parameters that may be apparent or strongly suggestive of changes in response to housing density alterations. These tests will then be incorporated into subsequent larger studies involving larger sample sizes and a wider range of different strains. The present study was devised as a preliminary experiment to examine the nature of animal responses to altered housing density. This information would enable specific hypotheses to be subsequently tested in confirmatory studies in the approach outlined in a previous work.[12] Second, by using the measures referred to earlier, we hoped to shed light on how laboratory mice respond to alterations in housing density. Third, we sought to raise awareness of the effects of increased housing density and ways to measure stress and to contribute to the ongoing discussion of these important issues. As a consequence of these aims, the current study was by necessity small in terms of sample size but broad in the range of physiologic parameters measured.[12]

Because the experiments were designed to detect possible alterations in physiologic characteristics and behavior in response to alterations in housing density, the only variable introduced into the study design was the number of mice in each cage. Changing this parameter not only changed the area of cage space available for each mouse but also altered the group size; variation in group size may have been a contributing factor to some of the outcomes observed.

We selected 2 common inbred strains, C57BL/6J (B6) and BALB/cJ (BALB), for the current study. We chose B6 mice because they are the most commonly used inbred strain in biomedical research.[24,27] We selected BALB mice because of their frequent use in previous density studies and because BALB males are more aggressive toward cagemates compared with many other inbred strains.[53] We reviewed previous studies to identify parameters that demonstrated differences likely attributable to changes in housing density (Figure 1). We selected a subset of these parameters for measurement in the current study. In addition, we included other measurements to detect whether increased housing density is associated with chronic stress. Our ultimate goal was to establish a set of quantitative parameters that could reliably assess changes in the wellbeing of laboratory mice. Mice were housed from weaning for 4 mo at 3 different densities, which was achieved by varying the number of mice per housing unit. The effects of different housing densities on various physiologic and behavioral parameters are presented and discussed.

Materials and Methods

Animals and housing conditions. Male and female mice of the C57BL/6J and BALB/cJ inbred strains (The Jackson Laboratory, Bar Harbor, ME) were used to determine the effect of different housing densities on physiologic and behavioral parameters of wellbeing. High housing density has been suggested to either cause or exacerbate the prevalence of barbering, to which B6 mice (especially females) are prone.[15] Male BALB mice are inherently moderately aggressive,[52] the evidence of which is (like barbering) easily observable; B6 mice are rarely aggressive.[23,53] All animals studied were SPF as supplied by the vendor as described previously.[46] All experimental and surgical procedures were approved by The Jackson Laboratory Institutional Animal Care and Use Committee.

Mice were housed in single-sex groups from weaning, at 3 wk for BALB and 4 wk for B6, and maintained in these groups for the duration of the experiments, 17 and 16 wk respectively. Mice were housed in positively pressurized, individually ventilated duplex cages (Thoren Caging Systems, Hazelton, PA). These consist of 2 adjacent pens, with each pen being 30.8 cm × 15.4 cm × 14.0 cm (12.13 in. × 6.05 in. × 5.53 in.) and a floor area of 333.5 cm^2 (51.7 $in.^2$). This caging system is the most commonly used system at our facility for production and experimental research. Each pen contains a wire-rod metal top that holds feed pellets and a water bottle. Animals had ad libitum access to bottled acidified water (pH 2.8 to 3.1) and feed pellets with 6% fat (NIH 31M, Purina Mills, Richmond, IN). Both sexes were set up with 4, 6, or 8 mice per pen, equivalent to 12.9, 8.6, and 6.5 $in.^2$/mouse (83.2, 55.5, and 41.9 cm^2 per mouse) respectively. There were 6 pens of each density. The minimum floor spaces recommended by the *Guide* for adult mice, that is those greater than 15 g or greater than 25 g body weight are 12 and 15 $in.^2$ per mouse (77.4 and 96.8 cm^2 per mouse), respectively.

The number of room air changes per hour was maintained at 15 ± 1, temperature at 22 ± 2 °C, and relative humidity at 45% ± 5%, with a 14:10-h light:dark cycle. Pens were maintained with positive pressure at 55 ± 5 air-changes per hour. Air pressure in the pen was determined by using an electronic micromanometer (Airdata Multimeter ADM870, Shortridge Instruments, Scottsdale, AZ). Standard bedding was white pine shavings (Crobb Box, Ellsworth, ME). Because the amount of bedding can influence in-cage NH_3 levels, the amount in each pen was standardized to a depth of 0.8 in. (21 ± 1 mm; 610 mL per cage). No nesting material beyond the pine shavings was added to any of the cages. Mice were moved to clean pens every 2 wk.

Study	Floor area per mouse (in.^{2})	Strain(s)	Age at onset (wk)	Duration (wk)	Parameters and measures (effect of reduced floor space)
Les, 1968	6.5, 8.7, 13, 17.3, 26, 52	C3H/HeJ, C57BL/6J, A/J, AKR/J, DBA/2,	4	6	**Growth rate (increased in A/J and C57BL/6J)** **Feed consumption (increased)**
Chvédoff and colleagues, 1980	11, 22, 44, 88	Crl, CD1, ICR, BR	Weaning	78–79	**Growth rate (increased)** **Feed and water consumption (increased)** Hematology (none) Serum biochemistry (none) Mortality (none) **Behavior (increased activity)** **Necropsy (increased chronic gastritis)**
Ortiz and colleagues, 1984	8.5, 17 3.4, 10, 20.5	OF1	3	4	Body (BW) and testis weight (none) Serum testosterone (none) *BW (decreased at 3.4 in.^{2})* Testis and adrenal gland wt (none)
Ortiz and colleagues, 1985	3.4, 10.3, 20.5 4.1, 20.5	OF1	3	4	*Growth rate (decreased)* Adrenal weight (none) Serum corticosterone (none) **Corticosterone response to acute stress (increased)** Adrenal weight (none) Serum corticosterone (none)
Peng and colleagues, 1989	7.5, 15, 30	BALB/cCrl	6–7	2	**Plasma corticosterone (increased)** Hematology (minor to none)
Peters and Festing, 1990		BALB/c, MF1	3	5	Growth rate (none, BALB/c; *decreased, MF1*) Aggression and injuries (none) Adrenal weight (***decreased, BALB/c;* increased, MF1**)
Fullwood and colleagues, 1998	5, 10, 15, 20	C57BL/6Crl	5–6	5	Growth rate (none) **Feed and water consumption (increased)** *Mortality (decreased)* **Adrenal weight (increased)** **Immune function (increased)** Hematology (none) **Plasma corticosterone (increased)**
McGlone and colleagues, 2001	5, 15, 20	BALB/cJ	3–4	6–7	Growth rate (none) Feed and water consumption (none) *Mortality (decreased)* Adrenal weight (none) Immune function (none or increased) Hematology (none) **Behavior (increased resting, feeding and grooming)**
Van Loo and colleagues, 2001	12.4, 19.4	BALB/cAnNCrlBr	7	14	Growth rate (none) **Feed and water consumption (increased)** **Behavior (increased agonistic encounters)** *Body condition (decreased fight wounds)* Serum testosterone (none) Urine corticosterone (none) Necropsy (none) Adrenal tyrosine hydroxylase activity (none)

Figure 1. Summary of published effects of increased housing density on mice. Parameters that increased with increased density (reduced floor space) are shown in bold, and those that decreased with increased density are in italics.

Physiologic measurements. ***Body weight and composition.*** At weaning, mice were assigned randomly to cages and housing density, at which time all mice were individually identified by ear-notching and numbered 1 through 4, 6, or 8 as appropriate to housing density. Prior to sample collection or test procedure, animals were allocated randomly to a procedure based on number, that numbered animal then underwent that test. Mice were weighed individually each week. Stress-mediated glucocorticoid release, together with age-related factors, affect body water and electrolyte content and distribution with subsequent changes in bone density.[40] Therefore we estimated changes in body composition and bone density by using dual-energy X-ray absorptiometry (DEXA; Lunar PIXImus, GE Healthcare, Waukesha, WI). At the study conclusion all mice were euthanized by CO_2 asphyxiation. Two mice from each pen were scanned by DEXA immediately after euthanasia. Another 2 mice from each pen were necropsied and had organs (heart, kidneys, adrenal glands, stomach, and gonads) removed, weighed, and fixed for histologic examination. Each organ also was examined for gross evidence of any disease process or change in size. Stomachs were examined by histology for evidence of gastritis or ulceration. To assess the percentages of the adrenal gland occupied by cortex

Smith and colleagues, 2004	5.7, 6.5, 8.6, 12.9	C57BL/6J	4	8	Growth rate (none) Feed and water consumption (none) Behavior (aggression none) Urinary testosterone (none) **Cage environment (increased NH_3, CO_2, temperature)** Nasal pathology (none)
Smith and colleagues, 2005	5.7, 6.5, 8.6, 12.9	BALB/cJ, FVB/NJ, NOD/LtJ	3–5	8	Growth rate (none) Feed and water consumption (none) **Behavior (increased aggression, FVB/NJ males at all densities)** Urinary testosterone (none) **Cage environment (increased NH_3, CO_2, temperature)**
Davidson and colleagues, 2007	5.6–33.5 6.5–39.2 9.4–54.5	Cr:SW	Breeders and pups until weaning at 3 wk	Not stated	Wean weight (none)
	13.4–22.3		Wean	6	Open field test, elevated plus maze, and light–dark exploration (suggestive of increased anxiety-like behavior with increased density)
Whitaker and colleagues, 2007	≤ 27.3 or ≤ 41.3 breeder trios and pups	C57BL/6Tac	8	4 litters	Litter size, pups weaned, percentage weaned, weight at weaning and interlitter interval (none)
	Wean 16.4–41		3	1–2	Elevated-plus maze, open-field activity test, and acoustic startle (minimal effects, suggesting that larger cages might lead to higher anxiety-like behavior)
O'Malley and colleagues, 2008	≤ 9.3	ICR	Primiparous females and pups	3	Dam weight; pups born per litter; percentage of pups weaned, sex ratio of pups; pup weights at 7, 14 and 21 d (none) *Nonadjusted pup growth rate (decreased)* Fecal corticosterone (none)
Laber and colleagues, 2008	7.5, 15, 37.5	Female BALB/cCrl C57BL/6Crl	Mature	9	*Growth rate (decreased, BALB/cCrl)* *Exploratory behavior (decreased)* **Plasma corticosterone (increased)** *Immune response, as measured by $CD4^+$ T cell counts (decreased–BALB/cCrl)*

Figure 1. Continued

and medulla, MetaMorph software (Molecular Devices, Downingtown, PA) was used to analyze midgland sections stained with hematoxylin and eosin. Table 1 lists all tests conducted, the numbers of animals tested for each strain, sex, and housing density as well as the frequency of each test.

Blood parameters. Approximately 200 µL blood was collected retroorbitally, after application of local anesthetic (tetracaine) to the eye, at 12 and 20 wk of age from as many as 6 animals per pen. Blood samples from individual mice were allocated to either hematology, biochemistry, or hormone analysis. Consequently, for most tests requiring blood collection, only 1 animal per cage was sampled. A full hemogram (RBC count, hemoglobin and RBC indices, total WBC count, and differential) was performed by using an automated analyzer (Advia 120 Hematology Analyzer, Bayer, Tarrytown, NY). The biochemistry panel, which included electrolytes, glucose, albumin, total protein, cholesterol, triglyceride, and thyroxine, was measured with an autoanalyzer (Synchron CX5 Delta Chemistry Autoanalyzer, Beckman Coulter, Brea, CA). Hormones measured included prolactin, thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormaone (LH) and testosterone. Plasma levels of these hormones are thought to act as stress indicators.[1,3,6,7,29] Plasma samples for prolactin and TSH assays were sent to Anilytics (Gaithersburg, MD), and those for testosterone, FSH, and LH to University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core [under NICHD (SCCPRR) grant U54HD28934; Charlottesville, VA]. Measurements were made by using either ELISA or radioimummoassay methods.

Fecal corticosterone metabolites. Fecal corticosterone metabolites (CM) were quantified by using an enzyme immunoassay, a noninvasive technique that reflects average blood levels of corticosterone. At the time of pen changing, 2 randomly selected mice from each cage were placed individually into a collection pen lined with absorbent paper to wick away urine for as long as 3 h. The fecal samples were collected from individual animals and analyzed separately. The mass of feces required for methanol extraction of CM is at least 0.05 g.[17,49,50] Because gastrointestinal transit time for CM in mice has been determined to be between 4 and 12 h on the basis of radioactive corticosterone injection studies,[50] we restricted the feces collection period to a maximum of 3 h to reduce the confounding influence of the stress of the collection method and animal handling on the CM measurements and to provide ample time to ensure sufficient quantity of feces for analysis. Because corticosterone needs to be cleared from the plasma by the liver before appearing in feces, the levels of CM better represent average, chronic values than do plasma corticosterone levels, which are more prone to acute variability.[48]

The collected fecal samples were analyzed for immunoreactive corticosterone metabolites by using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay. Details regarding

Table 1. Number of mice housed at different densities and the number and frequency of individual tests

	BALB/cJ						C57BL/6J					
	No. of female mice per pen			No. of male mice per pen			No. of female mice per pen			No. of male mice per pen		
	4	6	8	4	6	8	4	6	8	4	6	8
Total no. of pens	6	6	6	6	6	6	6	6	6	6	6	6
Total no. of mice	24	36	48	24	36	48	24	36	48	24	36	48
Body weight[a]	408	612	816	408	608	811	408	612	816	408	612	816
DEXA[b]	12	12	12	12	12	12	10	10	10	10	10	12
Organ exam[b]	12	12	12	12	12	12	10	10	10	10	10	12
Hematology[c]	10	12	12	10	12	12	7	11	11	7	11	12
Biochemistry[c]	9	12	12	10	12	11	7	11	11	7	11	12
Testosterone[c]	10	12	12	10	12	11	4	9	10	9	9	11
Prolactin[c]	7	12	12	7	12	12	7	11	11	7	11	12
Thyroid stimulating hormone[c]	7	12	12	8	12	12	8	11	11	8	11	12
Follicle stimulating hormone[c]	nd	nd	nd	nd	nd	nd	7	11	11	8	9	11
Luteinizing hormone[c]	nd	nd	nd	nd	nd	nd	8	12	11	7	11	12
Fecal cortisol metabolites[d]	28	30	30	28	30	28	20	20	16	20	18	16
Telemetry[e]	44	44	44	87	85	87	nd	nd	nd	76	76	76
Behavioral observations[a]	408	612	816	408	608	811	408	612	816	408	612	816
Behavioral tests[b]	12	12	12	12	12	12	6	6	4	6	6	4
Environmental monitoring[f]	1936	1937	1937	682	654	685	8266	4092	8271	12604	12458	3829

When more than 1 measurement was made, the number in the table is the total number of measurements, not the number at each time point; measurements were distributed as evenly as possible across time points.
nd, not done
[a]Weekly measurements
[b]Single measurement (at study end)
[c]Two measurements (12 and 20 wk of age)
[d]Four measurements for BALB/cJ (3, 10, 12, and 20 wk), and 3 measurements for C57BL/6J (10, 16, and 20 wk)
[e]Samples collected every 30 s, and data collapsed into daily 'bins,' thus representing more than 100,000 samples for each parameter
[f]BALB/cJ mice were sampled only 2 d per sample week, whereas the C57BL/6J mice were sampled daily each sample week; samples were collected only every other week, associated with cage changing.

development, biochemical characteristics, and biological validation of this assay have been described.[49,50] Before analysis fecal samples were homogenized and aliquots of 0.05 g were extracted with 1 mL of 80% methanol. A detailed description of the assay's performance has been published elsewhere.[50]

Briefly, the enzyme immunoassay used a double-antibody technique and was performed on microtiter plates coated with antirabbit IgG. After overnight incubation (at 4 °C) of standards (range, 0.8 to 200 pg/well) and samples with steroid antibody and biotinylated label, the plates were emptied, washed, and blotted dry before a streptavidin–horseradish peroxidase conjugate was added. After a 45-min incubation, plates were emptied, washed, and blotted dry. The substrate (tetramethylbenzidine) was added and incubated for another 45 min at 4 °C before the enzymatic reaction was stopped with 2 mol/L sulfuric acid. Then, the optical density (at 450 nm) was recorded by using an automatic plate reader, and the hormone concentrations were calculated. The intra- and interassay coefficients of variation were 9.1% and 14.0%, respectively.

Telemetry. To assess autonomic nervous system response to stress, cardiac electrical activity (for heart rate), body temperature, and general activity were recorded from a subset of mice by using a telemetric transponder (Heart rate E-Mitter, Mini-Mitter, Bend, OR) implanted into the abdominal cavity of 1 mouse in 1 pen per duplex pair.[51] Mice were anesthetized with intraperitoneal tribromoethanol (180 to 200 mg/kg; 2,2,2-tribromoethanol 99% in 2-methyl-2-butanol 99%, Acros Organics, Morris Plains, New Jersey, NJ) and, when they were at a surgical plane of anesthesia, were prepared by removal of much of the thoracic and abdominal fur, followed by alcohol and iodine scrubs. Mice also received carprofen (5 mg/kg IP) immediately prior to surgery. A 2-cm upper abdominal incision was made to allow insertion of the transmitter into the abdomen; the 2 cardiac leads then were tunneled subcutaneously to sit in the correct orientation to the heart, as outlined in the manufacturer's instruction manual. The leads were sutured into place with either 34-gauge stainless steel sutures (B6 mice) or 6-0 monofilament nonabsorbable sutures (Monosof, Covidien, Norwalk, CT; BALB mice). The abdominal muscle layer was closed with 5-0 absorbable suture (Dexon, Covidien), and 6-0 nonabsorbable suture (Monosof, Covidien) was used in the skin. In all, only 3 male B6 and 3 male and 2 female BALB mice from each density received implants. Female B6 mice did not receive implants because they did not reach a suitable body mass, according to the manufacturer's recommendations, until well into the study, at which point we felt any surgical intervention would have been too disruptive. A receiver platform (ER4000 Energizer Receiver, Mini-Mitter, Bend, OR) was placed under each pen, providing continuous data collection until the end of the experimental period. Male mice were implanted at 6 wk of age and female BALB mice at 9 wk, due to relative size constraints of mice and implants. Only 1 mouse per pair of duplex pens received an implant because the receivers could not distinguish between multiple transponders.

Behavior observations and tests. Mice were observed intermittently throughout the studies to assess behavior in general and aggressive behavior in particular. The main observations—for obvious signs of fighting or other aggressive behaviors— were

made after cage changing. Throughout the study, mice were observed for evidence of barbering. We categorized the 2 major types of barbering as 'barbering' (the removal or shortening of general body hairs from any and all parts of the body except the most rostral parts of the head, that is the muzzle) and 'whisker-picking,' where only vibrissae and immediately adjacent hairs were removed or shortened. Mice were scored for evidence of fight wounds and barbering at cage changing, that is every 2 wk, and at study end. Wound scoring was simple, recording the presence or absence of wounds generally from the most caudal parts of the animals. Barbering was scored by using a previously devised system that takes into account the region of the body affected, degree of hair loss, and condition of skin when exposed.[35] For this study, the only aspects of this scoring reported are prevalence and whether the distribution was on the animals' muzzle and/or on other parts of the body. Only the prevalence and distribution of barbering and whisker-picking at 20 wk of age is reported here because animals that were barbered never regrew their hair during the time course of this study.

At completion of each study, 2 mice from each pen were selected randomly for evaluation of anxiety-related behavior for 5 min on a modified hole board designed for this purpose.[37,38] Mice were videotaped as they roamed freely on a 24 in. × 24 in. (61 cm × 61 cm) board with a series of sixteen 1-in. (2.5-cm) holes in a 4 × 4 lattice in its center. The recordings were analyzed for: distance traveled by the mice, number of holes into which the mice poked their heads, and the number of times they did so. Also assessed from the recordings were the number of stretched attends (a measure of cautious exploration of a novel environment), general activity, and the numbers of fecal boli deposited and bouts of urination, which are considered to be expressions of anxiety.[16] Some of the hole-board and open-field analyses were automated by using a video-analysis program (Viewer, Biobserve, Bonn Germany) that also provided average mouse velocity.

Cage air monitoring. The pen microenvironment was monitored by using a multipoint gas sampler–doser and multipoint gas analyzer (models 1302 and 1303, Brüel and Kjær, Nærum, Denmark), capable of simultaneous measurement of NH_3, CO_2, water vapor (for relative humidity calculation), and temperature (accuracy was 2.5% for gases and water vapor and ± 0.2 °C for temperature). A probe was inserted low into each pen front for monitoring temperature, and an air-sampling line for gas analyses was inserted 1 in. above the bedding, into the breathing zone of the mice. All experimental pens were sampled continuously either for the second week of each 14-d pen change cycle (B6 mice) or throughout the 48-h period immediately prior to pen changing (BALB mice).

Statistical evaluation. The study's focus was on the comparative interaction of mice within each pen, therefore the experimental unit of measure for statistical analytical comparison was the pen. Data collected from animals in each pen was pooled, where appropriate, and mean data for each density calculated and compared. Because of the number of animals involved and the complexity of the study design, data for the 2 strains were collected sequentially rather than concurrently. Because each strain could be considered as an individual trial of a larger study, the interaction term for strain equates to that for trial. Furthermore, by combining the results from both strains, we increased the sample size so increasing the power to detect differences and it enabled us to examine the strain×density interactions. Parameters measured at a single time point (including body composition and organ weights) and formal behavioral testing were compared between density groups by using ANOVA. For single time point binary data (that is, terminal wounding and barbering scores), logistic regression for binary traits was used. Parameters measured at multiple time points (body weight, hematology, biochemistry, fecal CM, telemetry, and cage environment) were analyzed by repeated-measures multivariate ANOVA. For each trait, a full model was fitted that reflected the 4 factors (density, strain, sex, and age where appropriate) and all possible interactions. A backward model selection approach was used to achieve a final model, *P* values from which were used for significance tests for factors of interest. For all parameters (except where noted), the initial analyses combined data for all animals of both strains, both sexes, all densities, and all ages (time points), where relevant. When a result showed significant density-related variation, further analysis was performed to assess whether the differences were global or specific to an individual age, strain, sex, or density or combination of these parameters. The level of significance used was a *P* value of less than 0.05. Analyses were performed by using either JMP 7.0 (SAS Institute, Cary, NC) or SAS 9.1 (SAS Institute). The large environmental and telemetry datasets were assessed by collapsing data into weekly averages to eliminate brief fluctuations yet enable comparison of trends between density groups in a manner similar to that previously described.[32,33] Throughout the results section, values are presented in tables and figures as mean ± SEM; because of the large error bars in the figures, use of the SEM was chosen to improve clarity.

Results

Physiologic measurements. ***Body weight and composition.*** Throughout both studies, 3 animals died before study completion: 2 male BALB from the 6-per-pen group and 1 from the 8-per-pen group. Just prior to their deaths, these 3 animals were of average weight compared with their pen mates. All animals of both strains gained weight throughout the studies. Analysis of data from both strains revealed a significant difference ($F_{15,398}$ = 2.06; P = 0.0004) in body weight gain in mice housed at different densities. Animals housed at 8 per pen gained significantly less weight than did those in the lower density groups; this difference reached statistical significance in B6 males ($F_{15,397}$ = 1.85; P = 0.0270) and BALB males ($F_{15,397}$ = 3.07; P = 0.0001) and females ($F_{15,397}$ = 3.49; P < 0.0001; Figure 2 A, B).

Body composition as determined by DEXA analysis differed significantly only between densities for the combined results of both strains for bone mineral density (0.0541, 0.0534, and 0.0527 g/cm^2 for 4, 6, and 8 mice per pen, respectively; $F_{2,128}$ = 4.96; P = 0.0084). Bone area showed a significant strain×density interaction, with B6 mice at 6 per pen having significantly smaller bone area than those at higher and lower densities (8.83, 8.72, and 8.34 cm^2 for mice at 4, 8, and 6 per pen, respectively; $F_{2,126}$ = 4.38; P = 0.0146). For all DEXA parameters measured, there was a significant difference between strains, and for most parameters, a significant difference between male and female mice (data not shown).

Weights of dissected organs showed no differences between density groups (data not shown), although organ weights were all significantly different (P < 0.01 for all comparisons) between strains and sexes. The density×strain interaction showed that the percentage of adrenal gland occupied by corticosterone-producing cortex was significantly lower in BALB mice housed at 4 per pen (69.0% ± 1.02%, 74.0% ± 1.00%, and 72.9% ± 0.87% for mice at 4, 6, and 8 per pen, respectively) and in B6 housed at 6 per pen (74.8% ± 1.72,% 71.3% ± 1.25%, and 75.2% ± 1.78% at 4, 6, and 8 mice per pen, respectively; $F_{2,150}$ = 5.69; P = 0.0042). The stomachs from mice of both strains at all densities did not

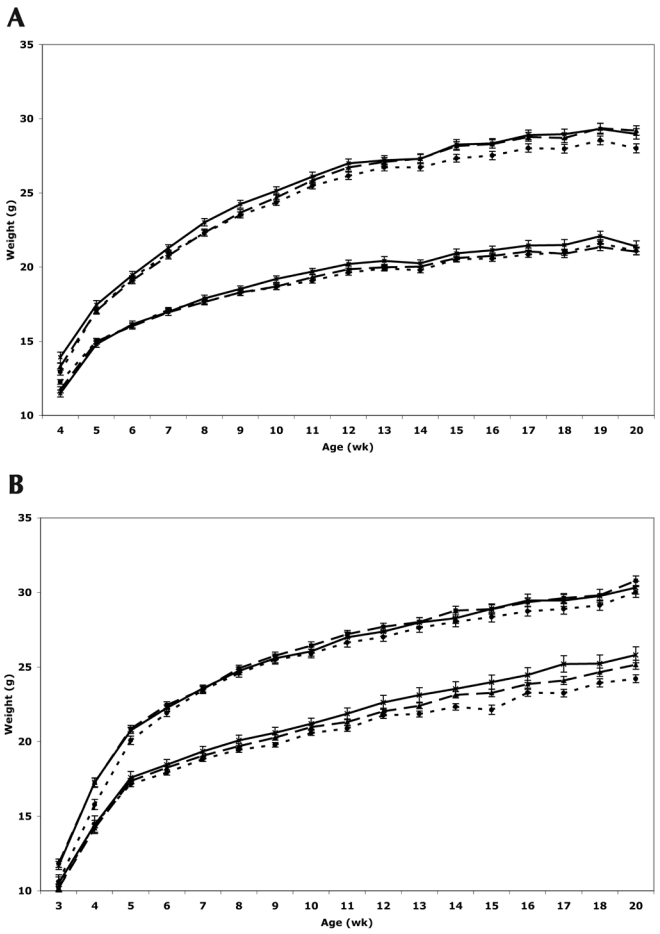

Figure 2. Mean weight-gain curves for (A) B6 and (B) BALB male (upper lines) and female mice (lower lines) housed at 3 densities [number of mice per pen: 4 (solid lines), 6 (dashed lines), and 8 (dotted lines)] from weaning to 20 wk of age. (A) B6 male mice housed at 4 and 6 per pen were significantly heavier than those housed at 8 per pen ($F_{15,397}$ = 1.85; P = 0.0270), whereas the weight of females (lower lines) did not differ by housing density ($F_{15,397}$ = 1.63; P = 0.0637). For BALB males (B, upper lines), mice housed at 4 and 6 per pen were significantly heavier than those at 8 per pen ($F_{15,397}$ = 3.07; P = 0.0001); females (lower lines) showed the same weight patterns ($F_{15,397}$ = 3.49; P < 0.0001).

show histologic evidence of ulceration. However, some animals of both strains at all 3 densities had evidence of chronic gastritis.

Blood parameters. Several hematologic parameters varied significantly with housing density, but these changes were not consistent across housing density or strain. The number of WBC was greater in mice housed at 8 per pen ($F_{2,53}$ = 4.53; P = 0.0153) at both time points, although the total WBC count decreased significantly for all densities of both strains over time ($F_{1,53}$ = 232.60; P < 0.0001). For the number of RBC, mice of the 2 strains housed at different densities responded differently over time; time×strain×density interaction ($F_{2,52}$ = 3.71; P = 0.0312). This difference was predominantly the result of decreased RBC in BALB mice housed at 8 per pen ($F_{1,52}$ = 6.69; P = 0.0125), whereas RBC in the B6 increased slightly at all densities. However, the hemoglobin concentration in BALB mice at 8 per pen did not change over time, whereas it increased in BALB mice housed at 4 and 6 per pen ($F_{1,51}$ = 3.76; P = 0.0300). The number and percentage of basophils changed differently over time in mice housed at 4 per pen compared with those at 6 and 8 per pen: the number of basophils decreased significantly ($F_{1,47}$ = 6.53; P = 0.0139), whereas the percentage of basophils actually increased in mice at 4 per pen but decreased slightly in mice at 6 and 8 per pen ($F_{1,51}$ = 11.44; P = 0.0014). The number and percentage of eosinophils changed differently over time between strains (data not shown) and housing densities (number of eosinophils: $F_{2,51}$ = 3.80; P = 0.0290; percentage of eosinophils: $F_{2,53}$ = 5.25; P = 0.0083); both the percentage and number of eosinophils increased in mice at 4 per pen, whereas the number decreased at the other densities with an associated slight increase in percentage, so the absolute number decreased while the relative number increased. Despite the statistically significant change in these few hematologic parameters, the magnitude of the changes in all instances was quite small. The only measured biochemical parameters to differ between densities and over time were the serum electrolytes sodium and chloride. Both electrolytes increased from 12 to 20 wk in mice housed at 8 per pen (Na: 12 wk, 156.2 ± 2.51; 20 wk, 158.1 ± 2.18 mmol/L, $F_{2,44}$ = 4.44; P = 0.0176; Cl: 12 wk, 118.1 ± 2.29; 20 wk, 123.2 ± 1.59 mmol/L, $F_{2,44}$ = 3.40; P = 0.0423), but Na decreased in mice at 4 and 6 per pen, and Cl either increased slightly or stayed essentially the same (data not shown).

None of the plasma hormones tested were significantly affected by density alone (data not shown).

Fecal corticosterone metabolites. Because samples for fecal CM were taken at different frequencies for the 2 strains, the response over time was analyzed separately for each strain. For both strains, female mice had significantly higher fecal CM than did male mice (BALB: $F_{3,24}$ = 7.89; P = 0.0008; B6: $F_{2,25}$ = 5.43; P = 0.0110; Figure 3 A). B6 mice housed at 8 per pen had significantly higher fecal CM overall (85.5, 89.7, and 106.6 ng per 0.05 g feces for 4, 6, and 8/pen respectively; $F_{2,26}$ = 4.20; P = 0.0261), which increased more over time than for those at lower densities ($F_{2,26}$ = 4.71; P = 0.0179; Figure 3 A). For BALB mice, CM approached significance over time with increased density ($F_{3,25}$ = 2.93; P = 0.0532).

Telemetry. Due to the staggered time of implantations as a result of the surgeon's schedule and the effect of recovery from surgery, analyses of telemetric data started 2 to 3 wk after implantation. This delay avoided highly variable results from individual animals due to the small number of animals receiving implants. Few significant density-related differences emerged from telemetric monitoring of body temperature, heart rate, and general activity. For the B6 male mice (females were not implanted because they were too small), activity was the 1 parameter to demonstrate significant differences; animals at 6 per pen were significantly more active than those at either 4 or 8 per pen (79.3, 72.8, and 70.7 arbitrary units for mice housed at 6, 4, and 8 per pen, respectively; $F_{2,212}$ = 13.24; P < 0.0001). Not surprisingly, the difference in activity between the dark and light periods was also highly significant (dark: 95.4 arbitrary units; light: 53.1 arbitrary units; $F_{1,212}$ = 878.38; P < 0.0001; Figure 4). Activity decreased significantly as the animals aged ($F_{12,212}$ = 4.42; P < 0.0001). In BALB mice, heart rate was lowest in those housed at 8 per pen; this difference reached significance only with male and female and light and dark data combined ($F_{8,19}$ = 5.03: P = 0.0018; Figure 5). The BALB heart rate was also significantly greater during dark than light (data not shown).

Behavioral observations and tests. Neither male nor female B6 mice showed gross evidence of fight wounds during or at the end of the study. However fighting among BALB males was evident at all densities during the study, with 25%, 8%, and 10% of male mice housed at 4, 6, and 8 per pen, respectively, having obvious signs of fight wounds at study end (Table 2). None of the wounds observed, which were restricted to the tail, were considered severe or appeared to affect the animals' overall health or body weight.

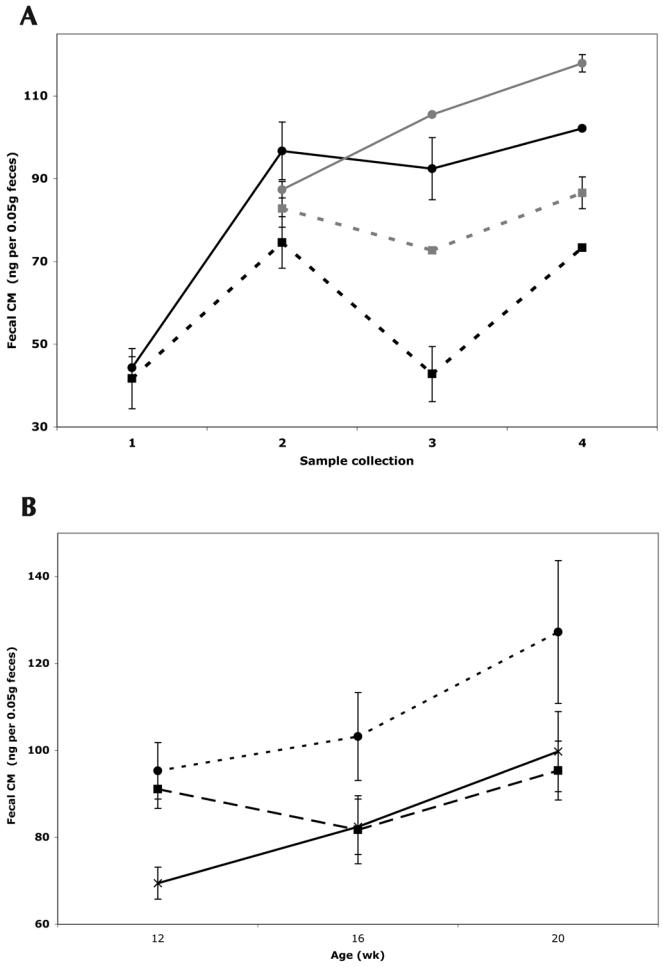

Figure 3. Fecal corticosterone metabolite (CM) concentrations for (A) both strains and sexes, showing changes over time; note that the *x* axis is not in weeks but sample collections, to allow better comparison of trends. For both strains, female mice (solid lines) had significantly higher fecal CM than did male mice (dashed lines; BALB (gray lines): $F_{3,24} = 7.89$; $P = 0.0008$; B6 (black lines): $F_{2,25} = 5.43$; $P = 0.0110$). (B) B6 mice housed at 8 per pen (dotted line) had significantly higher fecal CM for all time points combined (85.5, 89.7 and 106.6 ng per 0.05 g feces for 4, 6, and 8 mice per pen, respectively; $F_{2,26} = 4.20$; $P = 0.0261$); fecal CM also increased more over time among mice at 8 per cage than for animals at lower housing densities ($F_{2,26} = 4.71$; $P = 0.0179$).

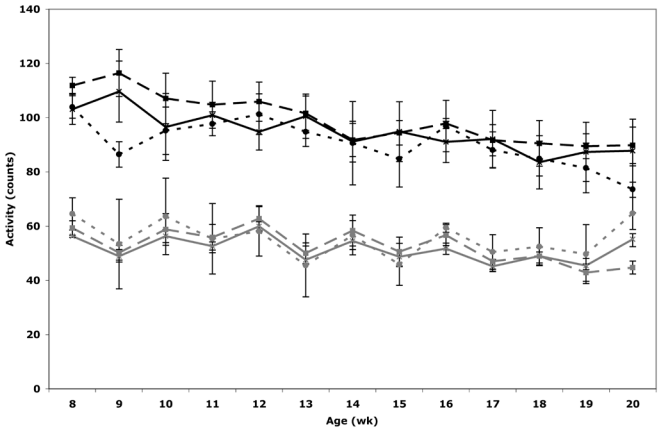

Figure 4. Example telemetric data for in-cage general activity of male C57BL/6J mice housed at 3 different densities. The graph starts at age 8 wk, after all animals had implants inserted and had recovered from surgery. The upper group of lines (black) shows the activity of male mice housed at 4 (solid lines), 6 (dashed lines), and 8 (dotted lines) per pen during dark hours, whereas the lower lines (gray) show their activity when the room lights were on. Overall, animals at 6 per pen were significantly more active than those at either 4 or 8 per pen (79.3, 72.8 and 70.7 arbitrary units for mice housed at 6, 4, and 8 per pen respectively; $F_{2,212} = 13.24$; $P < 0.0001$). Not surprisingly, the difference in activity between the dark and light periods was highly significant ($F_{1,212} = 878.38$; $P < 0.0001$).

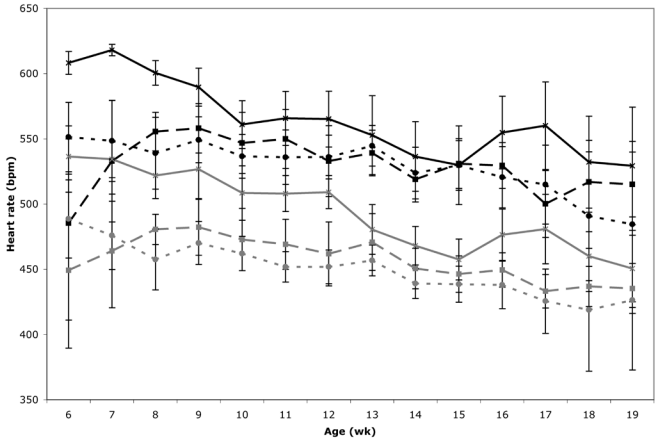

Figure 5. Combined telemetric data for heart rate (beats per minute; bpm) of male and female BALB/cJ mice; note female data are present only from week 9 onward because these mice needed more time to reach a suitable size for transmitter implantation. Data showing heart rate in the dark (black upper lines) and light (gray lower lines) for different housing densities: 4 per pen (solid lines), 6 per pen (dashed lines), and 8 per pen (dotted lines). Overall the heart rate tended to be higher in mice at 4 per pen and lower in those at 8 per pen and reached statistical significance when data for male and female mice and light and dark periods were combined ($F_{8,19} = 5.03$: $P = 0.0018$).

At 20 wk of age, the incidence of barbering across both strains was not different among mice housed at different densities (12.5%, 12.7%, and 12.0% respectively for 4, 6, and 8 mice per pen. However, strain had a highly significant effect on barbering: only 1 BALB animal showed signs of general body barbering. Therefore, this equal distribution of barbering across housing densities was effectively attributable to the B6 mice. Similarly a highly significant difference was detected in the incidence of barbering between sexes: 80% of barbered B6 mice were female ($\chi^2 = 50.40$; $P < 0.0001$), and 75% of whisker-picked B6 mice were female ($\chi^2 = 27.46$; $P < 0.0001$). For both strains considered together, whisker-picking at 20 wk of age had a higher incidence in those animals housed at 8 per pen (33.3%, 32.9%, and 50.3% for 4, 6, and 8 per pen, respectively; $\chi^2 = 13.04$; $P = 0.0015$). Whisker-picking and barbering were first observed in B6 mice of both sexes at all densities at 14 wk of age. By 20 wk, whisker-picking was significantly more common in B6 mice housed at 8 per pen in both sexes ($\chi^2 = 25.15$; $P < 0.0001$; Table 2). Whisker-picking first appeared in BALB male mice at 10 wk of age and in female mice at 12 wk of age; the incidence of whisker-picking at study end did not differ between BALB mice housed at different densities (Table 2).

For the exploratory hole-board open-field test, highly significant differences between the 2 strains were detected for several measured parameters, many of which suggest that BALB mice were more anxiety-prone than were B6 mice. The parameters affected include time spent in the central arena, latency to reach the perimeter, number of arena entries, number of stretched attends, and latency to the first hole visit. However, very few of these or other parameters showed differences in mice housed at different densities. For both strains, mice housed at 8 per pen made significantly fewer hole visits during their time on the

Table 2. Percentage (mean ± SEM) of animals barbered, whisker-picked, or wounded at 20 wk of age

		No. of animals assessed	% Barbered	% Whisker-picked	% Wounded
Density[a]					
	4	96	12.5 ± 3.39	33.3 ± 4.84	6.3 ± 2.48
	6	142	12.7 ± 2.80	33.1 ± 3.94	2.1 ± 1.21
	8	191	12.0 ± 2.36	50.3 ± 3.63	2.6 ± 1.16
Strain					
	BALB/cJ	213	0.5 ± 0.47	34.7 ± 3.26	6.6 ± 1.70
	C57BL/6J	216	24.1 ± 2.92	46.8 ± 3.40	0.0
Sex					
	Female	216	19.9 ± 2.72	49.1 ± 3.41	0.5 ± 0.46
	Male	213	4.7 ± 1.45	32.4 ± 3.20	6.1 ± 1.64
Strain and density					
	BALB/cJ , 4	48	0.0	35.4 ± 6.98	12.5 ± 4.82
	BALB/cJ , 6	70	1.4 ± 1.43	34.3 ± 5.65	4.3 ± 2.44
	BALB/cJ , 8	95	0.0	34.7 ± 4.91	5.3 ± 2.30
	C57BL/6J , 4	48	25.0 ± 6.32	31.3 ± 6.76	0.0
	C57BL/6J , 6	72	23.6 ± 5.04	31.9 ± 5.53	0.0
	C57BL/6J , 8	96	24.0 ± 4.38	65.6 ± 4.89	0.0

[a]Number of mice per pen.

exploratory hole-board open field (64, 68, and 56 visits for 4, 6, and 8 mice per pen respectively; $F_{2,98} = 4.97$; $P = 0.0088$), whereas number of visits did not differ between those housed at 4 and 6 mice pen. In addition, across both strains, mice housed at 4 per pen had more bouts of self-grooming than did those housed at 6 or 8 per pen (2.1, 1.0, and 1.4 grooming bouts for mice at 4, 6, and 8 per pen respectively; $F_{2,100} = 6.52$; $P = 0.0022$). When in the perimeter, B6 mice housed at 8 per pen reared less frequently than did those at 4 and 6 per pen (8.2, 7.4, and 5.0 rearings for mice at 4, 6, and 8 per pen respectively; $F_{2,96} = 4.95$; $P = 0.0090$). Mice housed at higher densities showed a trend toward defecating more while on the open field (3.4, 3.8, and 5.1 fecal boli for mice at 4, 6, and 8 per pen respectively; $F_{2,100} = 2.59$; $P = 0.0803$), whereas frequency of urination on the exploratory hole-board open field reached statistical significance as housing density increased (0.2, 0.4, and 0.6 urinations for mice at 4, 6, and 8 per pen respectively; $F_{2,100} = 4.11$; $P = 0.0192$).

Monitoring cage air. Pen NH_3 concentrations were relatively low throughout the period of study, with average values of less than 2.5 ppm for both strains and both sexes. However, 3 exceedingly high measurements were detected, which we considered to be due to animals urinating directly onto or near the air-sampling probe and therefore excluded from statistical analysis. The highest concentrations were almost exclusively in pens with 8 mice, although average values were not significantly different between mice housed at 6 and 8 per pen, whereas both were significantly greater than NH_3 concentration for mice housed at 4 per pen ($F_{1,463} = 21.29$; $P < 0.0001$). Ammonia concentration was significantly higher in pens housing BALB (BALB, 1.80 ± 0.04 ppm; B6, 1.68 ± 0.03 ppm; $F_{1,462} = 7.85$; $P = 0.0053$), greater for male mice (male, 1.78 ± 0.03 ppm; female, 1.67 ± 0.03 ppm; $F_{1,462} = 12.40$; $P = 0.0005$), and higher in pens at higher density (1.53 ± 0.03, 1.73 ± 0.03, and 1.92 ± 0.04 ppm for 4, 6, and 8 per pen respectively; $F_{2,462} = 33.02$; $P < 0.0001$) The mean CO_2 concentration for B6 mice was 3951 ± 77.5 ppm, whereas the pens of BALB mice had a mean concentration of 4525 ± 124.9 ppm. This strain difference in CO_2 concentration was significantly higher in BALB mice ($F_{1,444} = 18.42$; $P < 0.0001$). Across both strains, pens with increasing density of mice had significantly greater CO_2 concentration ($F_{2.444} = 37.02$; $P < 0.0001$).

Average pen temperatures generally remained between 22 and 28 °C for both strains but differed significantly between them (BALB, 25.6 ±0.14 °C; B6, 26.0 ± 0.09 °C; $F_{1,457} = 22.35$; $P < 0.0001$), although pens of 6 and 8 mice of both sexes and strains occasionally reached temperatures approaching 30 °C. Increased density cages had significantly increased temperatures: 24.6 ± 0.08, 26.2 ± 0.10, and 26.9 ± 0.12 °C for 4, 6, and 8 per pen, respectively ($F_{2,457} = 118.95$; $P < 0.0001$). Average pen relative humidity mostly stayed within the range of 37% to 55% (mean, 45.7% ± 0.24%) for B6 mice. Overall average relative humidity in the BALB pens (35.0% ± 0.38%) was significantly lower than for B6 pens ($F_{1,467} = 750.44$; $P < 0.0001$). Pens housing male BALB mice had a distinctly higher humidity range (30% to 46.5%; mean, 38.0% ± 0.48%) than did pens housing female BALB mice (24% to 39%; mean 32.34 ± 0.38%; $F_{1,467} = 82.96$; $P < 0.0001$). In contrast to other cage environmental measurements, cage relative humidity decreased as the number of mice in cages increased: cages of 4 mice had significantly higher relative humidity than did those with 6 and 8 mice (43.6% ± 0.57%, 41.71% ± 0.42%, and 41.1% ± 0.59%, respectively; $F_{2,467} = 6.95$; $P = 0.0011$).

Discussion

These experiments were carried out to address 2 questions: which parameters are most sensitive in detecting responses to housing density for use in future studies of mouse wellbeing, and whether increased housing densities would affect mouse phenotype. Three housing densities (as determined by the number of mice per cage) were examined: approximately the range recommended by the *Guide*; 50% greater than recommended, and 100% greater. The 2 increased densities represented decreases in floor space per mouse of 30% and 50%, respectively. The lowest density studied, 4 mice per pen, was wholly within

recommendations for the B6 female mice but at various time points during the study exceeded the guidelines by as much as 25% for B6 male and all BALB mice. Despite the small sample size used in this exploratory study, we found that several parameters were significantly different between animals housed at different densities. However not all of these observed differences were consistent between the 2 strains nor were they limited to mice of a particular housing density. For example, mice of both strains and both sexes housed at 8 per pen typically weighed less than did those at 4 and 6 per pen; the difference between those most densely housed and the others was more marked for the BALB. Similarly the few changes observed in hematologic parameters often differed (either in direction or degree of response) between the 2 strains over time. Such variability in results makes it difficult to draw absolute conclusions from this study. However, as we outlined in the introduction, this study was intended to refine methodology rather than provide robust conclusive answers to the many questions associated with the housing of laboratory mice. Regardless of this limitation (that is, sample size), the results presented suggest that there are limits to the number of mice that should be housed together if they are provided with a restricted amount of space in which to move around freely.

More importantly, the results demonstrate the value of certain tests over others in better defining the optimal housing density for laboratory mice. We feel that the most useful parameters to measure include body weight gain, adrenal gland size and percentage cortex, fecal CM, in-cage telemetry of activity and heart rate, and behavioral aspects such as incidence of barbering and whisker-picking, fighting, and formal tests of anxiety. Also of potential significance to future assessments of housing density is that some parameters measured here showed no statistically significant difference; whether this result reflected very small differences (thus requiring very large sample sizes for detection) or actual lack of effect due to increases in housing density is open to debate. Therefore, it is difficult to dismiss parameters—or ascribe great importance to—those parameters that did not appear to change in response to housing density. Does the lack of change mean that increased density has no effect on the particular aspect of physiology assessed, or did it not change due to insufficient sample size or sensitivity of the test? This dilemma cannot be resolved easily, especially by the current study with its limited sample size. Instead we have focused on those parameters that did change, because interpretation of these findings is less equivocal than for those that did not appear to change.

Some parameters from the study involving B6 mice were not measured during the study with BALB mice. Specifically, assays of follicle-stimulating and luteinizing hormones were omitted because subsequent discussions with reproductive biologists suggested that meaningful results could only be obtained if the stage of the estrus cycle was known at the time of sample collection. We did not attempt to determine the estrus cycle of the mice and so decided to exclude these measures from the BALB trial.

The mouse housing guidelines recommended in the *Guide for the Care and Use of Laboratory Animals* are based on cage floor space per mouse correlated to animal weight, from 6 $in.^2$ per mouse less than 10 g to more than 15 $in.^2$ for mice over 25 g. In the current studies, mice housed in groups of 4, 6, or 8 were provided respectively with 12.9, 8.6, or 6.5 $in.^2$ per mouse, regardless of body weight. Therefore, housing mice at 4 and 6 per pen complied with the *Guide*'s recommendations only for the first couple of weeks of the study, after which housing mice at 4 per pen complied only if mice weighed less than 25 g each. In practice, housing male mice at 4 per pen did not comply with guidelines by the midpoint of the study, whereas housing B6 females at this density complied throughout the study and could be considered *Guide*-relevant controls. At no stage did housing mice at 8 per pen comply with the recommendations in the *Guide*.

Mortality was not affected by housing density. However, mice housed at 8 per pen gained significantly less weight over the course of the study than did those at lower densities, regardless of strain or sex. Because all pens were provided with adequate supplies of food and water, lack of available nutrition is unlikely to have caused this difference. Perhaps there was a lack of sufficient time to gain access to feed and water, or perhaps a social hierarchy within these pens resulted in bullying of less-dominant animals. Previous studies have demonstrated that stress is capable of inducing catabolic effects in rodents, leading to reduced gains in body weight and body fat.[31] Because evidence of fighting by male BALB mice was distributed relatively evenly across density groups, fighting was unlikely to be a major factor in the weight differences observed. The significant difference in body weight at increased housing density seen in the present study is at odds with many of the other density studies,[13,18,26,30,34,42,45,46] perhaps because of their shorter duration (5 to 8 wk or less). However our data are in agreement with studies of similar duration.[2,5,25,39]

The housing density-related differences in body weight generally were not reflected by changes in body composition or organ weight, with the notable exception of the adrenal gland. Adrenal glands were larger, in both absolute weight and as a percentage of body mass, in female B6 mice at 8 per pen than in all other animals. In the B6 female mice, adrenal absolute size and percentage of cortex increased with increasing housing density, a trend that also occurred in the BALB male mice, but with much smaller differences between density groups. In BALB mice, histologic examination of the adrenal glands showed a significantly lower proportion of the adrenal occupied by the corticosterone-producing cortex in the least densely housed mice. Because corticosterone is the primary sustained-release stress hormone,[47] chronic stress might be expected to increase either overall gland size or the adrenal cortex in particular. Fecal corticosterone metabolites were significantly higher in the mice with the largest adrenal glands, suggesting a correlation between physiology and anatomic findings as well as between increased density and adrenal gland response. Together, these results strongly suggest that fecal CM measurements are a sensitive indicator of long-term effects of housing density and that increased housing density is associated with strain-specific stress responses. Increased plasma corticosterone levels with or without an associated increase in adrenal gland size were the most frequent endocrine change in other studies.[4,10,13,19,41]

Small but statistically significant differences in the number of WBC, RBC, basophils, and eosinophils and hemoglobin concentration were present either between density groups, strains, and time points but none were sufficiently consistent to suggest a physiologic mechanism. Total WBC numbers, especially neutrophils and lymphocytes, increase as part of an inflammatory response to injury or infection, neither of which were apparent in the present study but may have arisen as a result of fighting and injury among male mice. There were no consistent significant differences in total WBC, neutrophil, or lymphocyte counts between groups housed at different densities. Basophils most often increase in number in response to parasitic and other types of infection; animals were not examined specifi-

cally for presence of parasites, but all colonies at our facility are regularly screened for infectious agents and parasites with negative results. Eosinophils, which increased proportionately at all densities and only slightly in total number at 4 per pen, also tend to increase in response to parasitic infections. The few small changes in hematologic parameters suggest that in general, although relatively easy to perform, these tests are not of great value in assessing response to housing density.

The biochemical parameters that showed increases were plasma sodium and chloride concentrations. In normal physiologic responses, these 2 electrolytes tend to change in the same direction and to the same extent, so the current finding is relatively unremarkable. These 2 parameters increased the most over time in mice housed at 8 per pen; this change may have resulted from mild dehydration due to limited access to water. This response was most apparent in B6 mice. Although there were no statistically significant changes in any of the other biochemical parameters, any conclusions must be moderated by the fact that none of these animals were fasted prior to blood collection; food intake prior to measurement can falsely increase some of these parameters.[2] The oversight in not fasting the animals was only realized partway through the BALB trial as a result of analysis of the B6 results; we felt we should continue with the method that we started with to enable more reliable comparison between strains. Overall, hematology and serum biochemistry seem to be of little value in assessing the response of mice to increased housing density. Similar to the current findings, previous studies[5,13,26,41] found hematology and plasma biochemistry little affected as a direct result of increased housing density. Hematologic parameters in particular seem resistant to the influence of density differences: despite the decrease in WBC count over time, the response was similar for mice at all housing densities. Increased corticosterone secretion is known to decrease the total number of WBC.[2,13,26,34,41] The fact that mice at the 3 housing densities all showed significant decreases in WBC suggests that either some other mechanism led to the decrease or that all of the mice experienced stress- and corticosterone-induced leukopenia, an unlikely but perhaps plausible explanation. Plasma biochemistry showed no consistent relationship to housing density between and even within studies[2,13,26,34,41] and therefore cannot be considered a reliable indicator of response to housing density.

Plasma hormone concentrations were mostly unremarkable in response to changes in housing density. Prolactin has been reported to increase in response to stress and TSH to decrease (reviewed in references 3 and 25). The direction of the responses appeared to be dependent on the chronicity of the stress. In the current study, prolactin levels in female B6 and TSH levels in B6 males were slightly higher in mice housed at the lowest density. These findings suggest that measurement of prolactin and TSH is of limited value in assessing the chronic effects of housing density. Furthermore, prolactin levels in females vary with the stage of the reproductive cycle, thereby complicating accurate assessment from single time-point samples. Increased plasma and urinary testosterone levels have been associated with increased aggression in various strains, including BALB/cAnNCrlBR (reviewed in reference 46). Fighting among male mice, with obvious wounding, is the most common manifestation of aggression, but was infrequent in the present study, even in the more aggressive male BALB mice. The testosterone assay results in the current experiment were wide-ranging, which together with the small number of samples per cage, confound determination of whether significant within-cage differences were due to hierarchical dominance or aggressive behavior. For testosterone to be a useful test parameter, it may be necessary to sample all animals within each cage or to use a more sensitive assay (or both). Sampling more animals might also have detected more subtle differences in corticosterone levels. The other hormones assessed here did not appear to contribute to discrimination of housing density responses.

Although animals were not observed for prolonged periods, aspects of their penmate interactions can be inferred from assessment of the number and gross appearance of fight wounds as well the prevalence and extent of excessive grooming behaviors (that is, whisker-picking and barbering). Our results suggest that evidence of fighting is an unreliable effect of increasing housing density, both in fighting-prone as well as non-fighting-prone mice, at least as represented by the BALB and B6 strains. Published data[53-55] suggest dynamic interplay between group size and cage size together with strain effects in regard to aggression and fighting in male mice and as well effects on corticosterone and testosterone levels. Barbering was a more reliable marker of increasing housing density in our study, especially in B6 mice, and may be useful to monitor in future studies. Barbering has been interpreted as a repetitive abnormal behavior whose occurrence indicates increased social stress and decreased wellbeing.[14,15] However, this interpretation is not uniformly accepted,[21,44] and as our current results and others show,[21] barbering behavior is influenced strongly by strain. Although its propensity to occur in strains of black mice suggests a major genetic component, barbering still can be used as a strain-specific indicator for housing stress. In the current study, barbering and whisker-picking were not bound together inextricably as a single behavior. With 1 exception, BALB mice showed no barbering other than whisker-picking, and significantly more B6 only had whiskers but not body hair removed (Table 2). A review of earlier publications[15] referred in passing to a distinction between these behaviors; this differentiation requires further study to determine its importance. A related study[14] found no effect of housing density, group size, or dominance on the prevalence of barbering in C57BL/6J and related strains.

The telemetric data showed several trends but few significant housing density-related changes. This lack of significant differences between density groups perhaps was due to the few animals with implants per density group. The changes that were statistically significant were neither in the same strain nor in the same direction; activity was higher in BALB mice at 4 per pen, whereas heart rate was slower in B6 mice housed at 8 per pen. One would expect to find an increase in heart rate in the same animals in which activity was increased, as part of a normal physiologic response to exercise, but this pattern was not the case here. Perhaps the assay sensitivity or small sample size prevented detection of these changes, because if this response were accurate, then the mice at the lowest housing density would have had the highest activity levels and the highest heart rates. In future studies, the use of smaller implants in younger and smaller animals, combined with newer implant technology capable of monitoring of multiple implants from a single transceiver might better resolve whether this type of physiologic data from undisturbed animals discriminates subjects housed at different densities.

The results from the hole-board and open-field tests suggest that the mice housed at the highest density were more anxious when in a novel environment and were more reluctant to explore their new situation than were those at lower housing densities. More densely housed animals also tended to urinate and defecate more while grooming less, behaviors also indicative

of increased anxiety. These findings suggest it would be fruitful to include tests of anxiety in future studies of housing environment. To further deduce the nature of this anxiety, tests could also include the light–dark exploration test and the elevated plus maze or their variants, as well as tests with exposure to novel foods.[8]

Behavioral observations, both within the cage and with specific tests, appear to provide a robust means of distinguishing between density groups. In 1 study,[5] mice more densely housed had an increased level of physical activity, a finding opposite to that in another study,[26] in which although general activity was decreased, grooming behavior was increased, especially in female mice at the highest density. Regardless, the results of the previous study[26] were similar to our finding of increased barbering or whisker-picking of cagemates with increased density in both the B6 and BALB mice. Few other studies examining cage density effects have included formal behavioral tests, and of those that have, differences in basic study design preclude direct comparisons with our findings. One study[9] concluded that differences in housing density of preweaning and adolescent mice had little influence on anxiety-like behavior in young adult mice, in tests conducted with *Guide*-compliant housing densities. Another recent study[43] found that, compared with mice housed in isolation, those kept in crowded cages (groups of 7; the floor area per mouse was not stated) displayed more anxiety-like behavior. Despite a marked difference in study design, our present study similarly demonstrated that increasing housing density increased the display of anxiety-like behavior in both strains studied.

In broad agreement with other research,[45,46] our environmental data showed a strong relationship to housing density, except for relative humidity, which was independent of density. Pen temperature, NH_3, and CO_2 were well within recommendations of the *Guide*. NH_3 exposure was within OSHA's recommended maximal short-term exposure limit of 35 ppm.[36] At all densities studied, pen CO_2 increased to about 10 times ambient atmospheric levels but was more than 10-fold lower than the concentration stated by OSHA to be of immediate physiologic concern. Although increased CO_2 can have cardiovascular effects including increased blood pressure, vasodilatation, and increased heart rate, cardiac output telemetry failed to reveal any increases in heart rate at any density, suggesting that this level of exposure was unlikely of physiological concern to the mice. In sum, the data showed that although housing density clearly altered the pen environment, parameters remained within standard animal care guidelines and did not noticeably affect mouse physiology as determined by the tests used.

Because of the wide variety of parameters tested and the manner in which they differed in mice housed at different densities, together with the small sample size we used in this preliminary study, we caution against ascribing too much biologic significance to those parameters manifesting statistical significance. This caveat is reinforced by the fact that very few parameters displayed a linear response to altered housing density: in some instances, the animals housed at 6 per pen had a more marked response to a given parameter than did those housed at either extreme. Such variability makes it difficult to suggest a unifying response to increased housing density; however certain parameters are highly suggestive of significant responses to increased housing density and are worthy of inclusion in future confirmatory experiments. Factors for further analysis include body weight, adrenal gland weight and percentage cortex together with fecal CM, telemetry, in-cage behavior assessment (either directly or by proxy), and specific tests of behaviors indicative of anxiety and aggression or dominance.

In conclusion, mice housed from weaning until 20 wk of age at 3 different housing densities in the same size cages showed modest but statistically significant density-related differences in several phenotypic parameters. The most reliable parameters for discriminating different housing densities appear to be growth rate, adrenal gland size and cortical proportion, together with fecal metabolite corticosterone levels, in-cage behavior as assessed by telemetry, indicators of barbering, and formal behavioral testing for anxiety-like and exploratory behaviors. Despite statistically significant differences, in-cage monitoring of environmental parameters indicated that under most circumstances, the cage environment remained well within what are regarded as tolerable levels for mice. Increased sensitivity to the effects of housing density likely would be gained by further assessment of in-cage behaviors such as stereotypies (type and frequency), as well as aggressive interactions. These data would be best collected through careful, timed sampling using instantaneous and 1–0 sampling.[28] An expanded arsenal of behavioral tests to assess anxiety specifically would further enhance future studies. As stated in the introduction, the principal aim of the current study was to determine the most useful tests with which to assess the effects, if any, of increased housing density on laboratory mice. The current results also highlighted the need for increased numbers of pens (cages) of animals for sound statistical power and sampling of results. In some instances, results were suggestive of an effect but differences were not statistically significant. Some of these issues might be resolved with improvements in measurement technology, such as smaller, 'smarter' telemetric implants and assays requiring smaller volumes of blood or plasma, as well as a larger sample size.

The initial goals of this study were to determine the most appropriate tests to include in future, longer-term studies involving more inbred mouse strains and to add to current knowledge regarding the effects and suitability of housing mice at densities different to those prescribed by the *Guide for the Care and Use of Laboratory Animals*.[20] Judging from these data, we suggest that the density guidelines for housing mice could be increased (by 30% to 50%) to allow increased housing density with minimal or no negative effect on the wellbeing of inbred laboratory mice. Caveats to these conclusions include the response of different strains to the same conditions, as well as the effect of group size on social interactions regardless of cage size. This latter point was first highlighted in a 1968 study and has not been addressed clearly in studies since. Finally, the results of the present study suggest that housing mice at the currently recommended or slightly increased density has no or few significant effects on such diverse strains as B6 and BALB.

Acknowledgments

We thank Dr Oded Forman for help with the pathology; Dr Weidong Zhang and Benjamin King for assistance with the statistical analyses; and Drs Anne Peaston, Leonard Shultz, and Wesley Beamer for critical reading of this manuscript. This work was supported by funding from the Howard Hughes Medical Institute.

References

1. **Armario A, Lopez-Calderon A, Jolin T, Balasch J.** 1986. Response of anterior pituitary hormones to chronic stress. The specificity of adaptation. Neurosci Biobehav Rev **10:**245–250.
2. **Champy MF, Selloum M, Piard L, Zeitler V, Caradec C, Chambon P, Auwerx J.** 2004. Mouse functional genomics requires standardization of mouse handling and housing conditions. Mamm Genome **15:**768–783.

3. **Charmandari E, Tsigos C, Chrousos G.** 2005. Endocrinology of the stress response. Annu Rev Physiol **67:**259–284.
4. **Christian JJ.** 1961. Phenomena associated with population density. Proc Natl Acad Sci USA **47:**428–449.
5. **Chvedoff M, Clarke MR, Irisarri E, Faccini JM, Monro AM.** 1980. Effects of housing conditions on food intake, body weight, and spontaneous lesions in mice. A review of the literature and results of an 18-month study. Food Cosmet Toxicol **18:**517–522.
6. **Clark JD, Rager DR, Calpin JP.** 1997. Animal well-being. III. An overview of assessment. Lab Anim Sci **47:**580–585.
7. **Clark JD, Rager DR, Calpin JP.** 1997. Animal well-being. IV. Specific assessment criteria. Lab Anim Sci **47:**586–597.
8. **Crawley JN.** 2007. What's wrong with my mouse? Behavioral phenotyping of transgenic and knockout mice. Hoboken (NJ): Wiley–Interscience.
9. **Davidson LP, Chedester AL, Cole MN.** 2007. Effects of cage density on behavior in young adult mice. Comp Med **57:**355–359.
10. **Del Pup J, Palmes ED.** 1971. Effect of housing conditions on corticosterone levels in mice. Arch Environ Health **22:**493–495.
11. **Downing JE, Miyan JA.** 2000. Neural immunoregulation: emerging roles for nerves in immune homeostasis and disease. Immunol Today **21:**281–289.
12. **Festing MF.** 2006. Design and statistical methods in studies using animal models of development. ILAR J **47:**5–14.
13. **Fullwood S, Hicks TA, Brown JC, Norman RL, McGlone JJ.** 1998. Floor space needs for laboratory mice: C57BL/6 males in solid-bottom cages with bedding. ILAR J **39:**29–36.
14. **Garner JP, Dufour B, Gregg LE, Weisker SM, Mench JA.** 2004. Social and husbandry factors affecting the prevalence and severity of barbering ('whisker trimming') by laboratory mice. Appl Anim Behav Sci **89:**263–282.
15. **Garner JP, Weisker SM, Dufour B, Mench JA.** 2004. Barbering (fur and whisker trimming) by laboratory mice as a model of human trichotillomania and obsessive–compulsive spectrum disorders. Comp Med **54:**216–224.
16. **Grewal SS, Shepherd JK, Bill DJ, Fletcher A, Dourish CT.** 1997. Behavioural and pharmacological characterisation of the canopy stretched attend posture test as a model of anxiety in mice and rats. Psychopharmacology (Berl) **133:**29–38.
17. **Harper JM, Austad SN.** 2000. Fecal glucocorticoids: a noninvasive method of measuring adrenal activity in wild and captive rodents. Physiol Biochem Zool **73:**12–22.
18. **Ikeno Y, Hubbard GB, Lee S, Richardson A, Strong R, Diaz V, Nelson JF.** 2005. Housing density does not influence the longevity effect of calorie restriction. J Gerontol A Biol Sci Med Sci **60:**1510–1517.
19. **Ishida H, Mitsui K, Nukaya H, Matsumoto K, Tsuji K.** 2003. Study of active substances involved in skin dysfunction induced by crowding stress. I. Effect of crowding and isolation on some physiological variables, skin function and skin blood perfusion in hairless mice. Biol Pharm Bull **26:**170–181.
20. **Institute of Laboratory Animal Resources.** 1996. Guide for the care and use of laboratory animals. Washington (DC): National Academy Press.
21. **Kalueff AV, Minasyan A, Keisala T, Shah ZH, Tuohimaa P**. 2006. Hair barbering in mice: implications for neurobehavioural research. Behav Processes **71:**8–15.
22. **Koolhaas JM, Baumans V, Blom HJM, Von Holst D, Timmermans PJA, Wiepkema PR.** 1993. Behaviour, stress, and well-being. In: Van Zutphen LFM, Baumans V, and Beynen AC, editors. Principles of laboratory animal science: a contribution to the humane use and care of animals and to the quality of experimental results. Amsterdam (Netherlands): Elsevier Science BV.
23. **The Jackson Laboratory.** Inbred strains of mice and rats at the Mouse Genome Informatics website. [Cited 21 Feb 2008]. Available at http://www.informatics.jax.org/external/festing/search_form.cgi.
24. **The Jackson Laboratory**. C57BL/6J. JAX mice database. [Cited 21 Feb 2008] Available at http://www.jaxmice.jax.org/strain/000664.html.
25. **Les EP.** 1968. Cage population density and efficiency of feed utilization in inbred mice. Lab Anim Care **18:**305–313.
26. **Les EP.** 1968. Environmental factors influencing body weight of C57BL/6J and DBA/2J mice. Lab Anim Care **18:**623–625.
27. **Malakoff D.** 2000. The rise of the mouse: biomedicine's model mammal. Science **288:**248–253.
28. **Martin P, Bateson P.** 1993. Measuring behaviour: an introductory guide. Cambridge (UK): Cambridge University Press.
29. **Matteri RL, Carroll JA, Dyer CJ.** 2000. Neuroendocrine responses to stress. In: Moberg GP, Mench JA, editors. The biology of animal stress: basic principles and implications for animal welfare. Wallingford (UK): CABI Publishing.
30. **McGlone JJ, Anderson DL, Norman RL.** 2001. Floor space needs for laboratory mice: BALB/cJ males or females in solid-bottom cages with bedding. Contemp Top Lab Anim Sci **40:**21–25.
31. **Michel C, Duclos M, Cabanac M, Richard D**. Chronic stress reduces body fat content in both obesity-prone and obesity-resistant strains of mice. Horm Behav **48:**172–179.
32. **Nadziejko C, Chi Chen L, Nadas A, Hwang JS.** 2004. The 'Fishing License' method for analysing the time course of effects in repeated measurements. Stat Med **23:**1399–1411.
33. **Nadziejko C, Fang K, Chen LC, Gordon T, Nadas A.** 2002. Quantitative analysis of cardiac data from rats monitored by telemetry: reducing within- and between-animal variability. Cardiovasc Toxicol **2:**237–244.
34. **Naidu S, Winget CM, Jenner JW, Mele G, Holley DC.** 1995. Effects of housing density on mouse physiology and behavior in the NASA Animal Enclosure Module simulators. J Gravit Physiol **2:**P140.
35. **Nicholson A, Malcolm RD, Russ P.** 2009. A simple system for scoring degree and severity of barbering in mice. Unpublished data.
36. **Occupational Safety and Health Administration.** Ammonia in workplace atmospheres—solid sorbent. [Cited 03 Feb 2008] Available at http://www.osha.gov/dts/sltc/methods/inorganic/id188/id188.html.
37. **Ohl F, Roedel A, Binder E, Holsboer F.** 2003. Impact of high and low anxiety on cognitive performance in a modified hole board test in C57BL/6 and DBA/2 mice. Eur J Neurosci **17:**128–136.
38. **Ohl F, Sillaber I, Binder E, Keck M, Holsboer F.** 2001. Differential analysis of behavior and diazepam-induced alterations in C57BL/6N and BALB/c mice using the modified hole board test. J Psychiatr Res **35:**147–154.
39. **Ortiz R, Armario A, Castellanos JM.** 1984. Postweaning differential housing and testosterone secretion in male mice. Experientia **40:**1428–1429.
40. **Pedersen WA, Wan R, Mattson MP.** 2001. Impact of aging on stress-responsive neuroendocrine systems. Mech Ageing Dev **122:**963–983.
41. **Peng X, Lang CM, Drozdowicz CK, Ohlsson-Wilhelm BM.** 1989. Effect of cage population density on plasma corticosterone and peripheral lymphocyte populations of laboratory mice. Lab Anim **23:**302–306.
42. **Peters A, Festing M.** 1990. Population density and growth rate in laboratory mice. Lab Anim **24:**273–279.
43. **Reiss D, Wolter-Sutter A, Krezel W, Ouagazzal AM.** 2007. Effects of social crowding on emotionality and expression of hippocampal nociceptin/orphanin FQ system transcripts in mice. Behav Brain Res **184:**167–173.
44. **Sarna J, Dyck R, Whishaw I.** 2000. The Dalila effect: C57BL6 mice barber whiskers by plucking. Behav Brain Res **108:**39–45.
45. **Smith AL, Mabus SL, Muir C, Woo Y.** 2005. Effects of housing density and cage floor space on 3 strains of young adult inbred mice. Comp Med **55:**368–376.
46. **Smith AL, Mabus SL, Stockwell JD, Muir C.** 2004. Effects of housing density and cage floor space on C57BL/6J mice. Comp Med **54:**656–663.
47. **Spackman DH, Riley V.** 1978. Corticosterone concentrations in the mouse. Science **200:**87.
48. **Touma C, Palme R.** 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann N Y Acad Sci **1046:**54–74.

49. **Touma C, Palme R, Sachser N.** 2004. Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. Horm Behav **45:**10–22.
50. **Touma C, Sachser N, Mostl E, Palme R.** 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocrinol **130:**267–278.
51. **van Bogaert MJ, Groenink L, Oosting RS, Westphal KG, van der Gugten J, Olivier B.** 2006. Mouse strain differences in autonomic responses to stress. Genes Brain Behav **5:**139–149.
52. **Van Loo PL, Van der Meer E, Kruitwagen CLJJ, Koolhaas JM, Van Zutphen LFM, Baumans V.** 2003. Strain-specific aggressive behavior of male mice submitted to different husbandry procedures. Aggress Behav **29:**69–80.
53. **Van Loo PL, Van Zutphen LF, Baumans V.** 2003. Male management: coping with aggression problems in male laboratory mice. Lab Anim **37:**300–313.
54. **Van Loo PLP, Kruitwagen CLJJ, Koolhaas JM, Van de Weerd HA, Van Zutphen LFM, Baumans V.** 2002. Influence of cage enrichment on aggressive behaviour and physiological parameters in male mice. Appl Anim Behav Sci **76:**65–81.
55. **Van Loo PLP, Mol JA, Koolhaas JM, Van Zutphen BFM, Baumans V.** 2001. Modulation of aggression in male mice: influence of group size and cage size. Physiol Behav **72:**675–683.

Journal of the American Association for Laboratory Animal Science
Copyright 2010
by the American Association for Laboratory Animal Science

Vol 49, No 2
March 2010
Pages 136–137

Letters to the Editor

The Response of C57BL/6J and BALB/cJ Mice to Increased Housing Density

Dear Editor,

We are writing in regard to the article by Nicholson A and colleagues.[3] We believe that the data presented do not support the primary conclusion drawn by the authors, namely, that housing density can be increased significantly over current *Guide* standards[2] with few if any adverse effects. We also note that the conclusions stated in the abstract do not coincide with those in the text. The abstract states that the findings support a 50% to 100% increase over current *Guide* standards,[2] whereas the discussion mentions a 30% to 50% increase. Although this may stem from confusion about the number of mice per cage versus the amount of floor space per mouse, it is an important distinction that requires clarification by the authors.

Rather than supporting an increase in housing density, the data presented suggest that significant increases in density have adverse effects. A 100% increase above the current *Guide* standards[2] appeared detrimental to the mice, based on criteria that the authors determined were most significant for determining wellbeing. These criteria included weight gain, adrenal gland size and percentage cortex, fecal corticosterone, in-cage telemetry of activity and heart rate, and behavioral aspects such as incidence of barbering and whisker-picking, fighting, and formal tests of anxiety. Many of these parameters changed with increasing density and were interpreted by the authors to be indicative of chronic stress or anxiety. Similar findings were reported by many of the papers cited in Figure 1 of the article.[3] Thus, it is unclear why the authors would claim that housing density could be increased as much as 100% without affecting overall wellbeing.

The validity of this conclusion is also called into question by the fact that the stated goal of the study was to serve as a preliminary experiment that would enable the testing of specific hypotheses in subsequent larger studies. The study was not designed to determine whether housing density could be increased; rather, the primary goal was to determine what parameters should be incorporated into future experiments. This tends to weaken any conclusions about housing density per se.

The authors emphasize in the discussion that more extensive behavioral measurements are important to determining appropriate housing density. We strongly concur and would argue that simple strategies, such as monitoring for gross evidence of negative behavior (fighting, barbering, and whisker pulling), are necessary but not sufficient for assessing wellbeing. More refined behavioral testing would add substantially to understanding the optimal housing requirements for mice. Assessments of time budget, preference testing, and demand (how hard the animal will work to obtain the preference) would shed light on how animals use the space available to them. Behavioral measurements of anxiety in the presence of a strange setting or unknown animals would help clarify the extent to which high density housing is associated with stress responses.

This topic was discussed in depth at the 2006 National AALAS meeting and later published in the November 2007 issue of Lab Animal.[1] We believe for fairness to the reader and the laboratory animal community the author should reference this publication since in that forum similar studies from the same laboratory were criticized for weaknesses in experimental design and data interpretation.

We would also point out that the issues at hand are not strictly scientific in their reach. There are ethical concerns and public perceptions that should influence the discussion. Providing sufficient space to permit species-typical activities and social interactions is an ethical imperative. Even if studies suggest that increased housing density is devoid of negative effects, implementation of high-density housing may lead to the public perception that animals are crowded into an unacceptably small space. This perception may well diminish public confidence in the proposition that the welfare of research animals is properly assured.

Sincerely,
Charmaine Foltz, DVM, DACLAM
Director, Division of Veterinary Resources
National Institutes of Health

David DeLong
Chief, Veterinary Medical Unit
VA Medical Center (151)

References

1. **Foltz C, Carbone L, DeLong D, Rollin B, Van Loo P, Whitaker J, Wolff A.** 2007. Considerations for determining optimal mouse caging density. Lab Anim **36:**40–48.
2. **Institute for Laboratory Animal Research.** 1996. Guide for the care and use of laboratory animals. Washington (DC): National Academies Press.
3. **Nicholson A, Malcolm RD, Russ PL, Cough K, Touma C, Palme R, Wiles MV.** 2009. The response of C57BL/6J and BALB/cJ mice to increased housing density. J Am Assoc Lab Anim Sci **48:**740–753.

Response to Drs Foltz and DeLong's Letter to the Editor:

We thank Drs Foltz and DeLong for their comments bringing to our attention the discrepancy between the data presented in the abstract and our conclusions,[3] and we regret any confusion this may have caused in the minds of readers. To clarify the ambiguity, we include here a table explicitly relating the housing densities used in our study to those recommended in the *Guide*.[2]

Body weight	Recommended no. of mice per pen*	Relative increase in housing density for 4, 6 or 8 mice per pen[3]		
		4	6	8
> 25 g	3	33%	100%	166%
15-25 g	4	0%	50%	100%
10-15 g	6		0%	33%
< 10 g	8			0%

*Number of mice of different weight categories per pen in duplex cages of 51.7 in.[2] in accordance with the recommendations of the *Guide*.

The data for the largest animals (that is, those > 25 g) showed that mice housed at 4 or 6 per pen demonstrated no significant differences for most of the parameters measured. In fact, none of the female C57BL/6J mice attained a weight > 25 g during the study, so they remained within the *Guide* recommendations throughout. No significant differences were noted between these female C57BL/6J mice and other mice housed at 4 or 6 per pen. Therefore, we concluded that the housing density of mice could be increased by up to 100%, to 4 or 6 per pen, with no apparent ill effect on wellbeing as reflected in the wide range of tests employed in our study. This did not appear to be the

case for animals housed at 8 per pen.

Although we suggested, as part of our conclusions, that the housing density could be increased beyond that recommended in the *Guide*, at no point did we advocate for such an increase. In fact, on p 751 in the article, we cautioned against ascribing too much biologic significance to those parameters manifesting statistical significance.

This caveat is reinforced by the fact that very few parameters displayed a linear response to altered housing density. Such variability makes it difficult to suggest a unifying response to increased housing density; however, certain parameters are highly suggestive of significant responses to increased housing density and are worthy of inclusion in future confirmatory experiments.[3]

Furthermore, the most detrimental response to increased housing density was in those mice housed at 8 per pen. Some negative responses were seen relatively early on in the study before the mice exceeded 25 g in weight. For example, the growth curves presented in Figure 2 on p 746 suggest that group size may have had a greater role than floor area by itself.[3] The question of group size versus cage size has been raised by several other studies referred to in our paper but none seems to have clearly distinguished between these two environmental factors.

Our study was preliminary, as our primary aim was to assess a range of test parameters to determine which were most helpful in detecting differences in the response of mice to altered housing densities. Indeed, we were able to conclude that some tests were useful and others were not. In addition, our data did not indicate that housing density increases were uniformly detrimental to the animals, and we stand by this conclusion. At the same time we strongly agree with the correspondents' calls for more extensive behavioral testing in future studies and are fully cognizant of the ethical obligations and emotional (public perception) aspects of the welfare of animals used for research. These considerations, together with the study limitations that we pointed out (that is, sample size, lack of assessment of the impact of environmental enrichment, and testing of only animals housed in single-sex groups) further induced us to recommend caution in interpretation of our results. However, we consider the data interesting and believe that our cautiously drawn conclusions are reasonable and provide food for thought about mouse wellbeing and how to assess it.

Finally we take issue with the correspondents' reference to Foltz and colleagues,[1] in which previous studies from The Jackson Laboratory (TJL) were criticized for some of the assumptions made and resulting conclusions reached. First, none of the authors in the current study were involved in the previous studies although most of us have worked at TJL. Second, we did not feel that overall the comments[1] were directly relevant to nor contributed to the assessment of our data because we included many more physiological and behavioral tests than the earlier TJL studies. We did not include all the behavioral tests suggested[1] because as with many researchers, we had to make decisions about, and restrict ourselves to, those tests we felt we could best perform to gain reliable data within our limited budget. Comprehensive housing studies that incorporate factors such as strain differences, effect of environmental enrichment, more extensive in- and out-of-cage behavioral testing, as suggested by the correspondents, are necessary, highly desirable, and expensive to conduct. Securing funding for such complex, large-scale studies is extremely difficult, because despite the importance of this area of research, with its potential for wide-ranging impact on much other research, those funding agencies with adequate funds appear to focus their support elsewhere.

In conclusion, we agree in large part with the suggestions of Foltz and DeLong but contend that the differences in our interpretation of the data stem from differences in emphasis. Our emphasis was to identify reliable and valid parameters for evaluation of responses to changes in housing density for use in future studies. Despite our findings suggesting that increased housing density may have no or limited apparent detrimental impact on the 2 strains of mice studied, we did not advocate implementing such an increase. We are well aware of the need for further studies before concluding that one way or the other is preferable or even adequate. Our intentions were to contribute to this important and ongoing debate concerning laboratory mouse welfare.

Respectfully yours,

Anthony Nicholson, BVSc, PhD, DACVA
Nicholson Anesthesia and Comparative Phenotyping Consultancy

Rachel D Malcolm, MS
The Jackson Laboratory

Chadi Touma, Dr rer nat, Dipl Biol
Department of Psychoneuroendocrinology
Max Planck Institute of Psychiatry
Munich, Germany

Rupert Palme, DVM, PhD
Department of Biomedical Sciences/Biochemistry
University of Veterinary Medicine
Vienna, Austria

Michael V Wiles, PhD
Senior Director, Technology Evaluation and Development
The Jackson Laboratory

References

1. **Foltz C, Carbone L, DeLong D, Rollin B, Van Loo P, Whitaker J, Wolff A.** 2007. Considerations for determining optimal mouse caging density. Lab Anim **36:**40–48.
2. **Institute for Laboratory Animal Research.** 1996. Guide for the care and use of laboratory animals. Washington (DC): National Academies Press.
3. **Nicholson A, Malcolm RD, Russ PL, Cough K, Touma C, Palme R, Wiles MV.** 2009. The response of C57BL/6J and BALB/cJ mice to increased housing density. J Am Assoc Lab Anim Sci **48:**740–753.

Letters to the Editor

Letters discuss material published in *JAALAS* in the previous 3 issues. They can be submitted through email (journals@aalas.org) or by regular mail (9190 Crestwyn Hills Dr, Memphis, TN 38125). Letters are not necessarily acknowledged upon receipt nor are the authors necessarily consulted before publication. Whether published in full or part, letters are subject to editing for clarity and space. The authors of the cited article will generally be given an opportunity to respond in the same issue in which the letter is published.