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Effects of Housing Density on Weight Gain, Immune Function, Behavior, and Plasma Corticosterone Concentrations in BALB/c and C57BL/6 Mice

Kathy Laber,[1,2*] Lynn M Veatch,[1,2] Marcelo F Lopez,[1] Jennifer K Mulligan,[1] and Deanne MR Lathers[1,2]

The *Guide for the Care and Use of Laboratory Animals* contains recommended housing densities for rodent species that are commonly used by the scientific community. However, at the time of the *Guide's* publication, housing density recommendations were based heavily on the professional judgment of qualified scientists. Some scientists therefore question whether rodents can be housed at greater densities, whereas others wonder whether the space currently provided for rodents is sufficient. The present study was designed to determine the effect of housing adult female BALB/c- and C57BL/6-mice in standard 75-in^2 (484-cm^2) ventilated cages at various housing densities (n = 2, 5, and 10 mice/cage). Measures of weight gain, plasma corticosterone, behavior, and immune parameters were evaluated at 7, 28, and 70 d after housing allocation. Housing BALB/c mice at 10/cage had negative effects on weight gain, corticosterone, behavior, and immune parameters. Housing C57BL/6 mice at 10/cage did not affect immune function or weight gain, although behavior and corticosterone showed statistical trends implying a negative effect Differences associated with housing densities of 2 and 5 mice/cage were less robust for all variables measured. We conclude that housing female BALB/c mice at 10 mice/cage (that is, at twice the *Guide*-recommended density) affects their physiology. We also conclude that mice vary in their responses in the parameters measured. These observations support the conclusion that it will be extremely challenging to scientifically determine an optimal cage density standard that can be uniformly applied across all mouse strains.

The housing of mice at caging densities that are optimal for both the needs of the scientific community and the animals' wellbeing is a highly desirable goal for both investigators and regulatory agencies. Scientists must ensure that caging density does not create uncontrollable scientific variables, maintains the health of the animals, and supports an appropriate study design regardless of cost. The regulatory agencies, who respond to the public's demand for optimizing animal welfare, need to ensure that the caging density minimizes the distress that may occur due to either overcrowding or isolation.

The standard currently used in the US for rodent housing densities—*The Guide for the Care and Use of Laboratory Animals*—acknowledges that an animal's space needs are complex but specifies that at a minimum, an animal must have enough space to turn around to express normal postural adjustments and have enough clean-bedded or unobstructed area to move and rest.[27] These performance parameters help to support the current *Guide* recommendations for housing mice according to the animals' weights and the number of animals per cage. In other countries including Canada and those of the Council of Europe (which comprises 40 member states), housing densities for rodents have similarly been based primarily on professional judgment, with the resulting guidelines indicating even more space than what the *Guide* recommends.[3,13]

Recent publications have challenged the recommended housing standards by determining physiologic variables in diverse strains of mice. Two studies housing animals at the *Guide*-recommended space allowances for mice weighing more than 25 g (15 in^2, 96.8 cm^2)[14,24,27] demonstrated immune suppression in both male and female mice as compared with housing mice in smaller spaces (5 in^2, 32.3 cm^2). However, cage size and corticosterone levels in male C57BL/6 mice were inversely related, implying that animals housed in less space experience more stress.[14] Other studies in male mice noted that prolonged individual housing altered immune parameters (that is, increased natural killer-cell activity, macrophage activation, and lymphocyte proliferation in response to phytohemaglutinin).[17,18,29] A thorough understanding of the effect of cage density on the immunologic profile of mice is critical to numerous areas of research, including cancer, autoimmunity, immunology, and hematology. If mice housed at higher caging densities have similar immune functions as those housed according to *Guide* recommendations, monetary and space restraints faced by investigators could be alleviated.

We selected C57BL/6 and BALB/c mice for the studies described here because of their frequent use in scientific research and because they diverge in measures of anxiety, memory,[6,10,16,28,33] and immunologic profile.[8,15,20,21] Several prior studies have demonstrated that C57BL/6 mice are stress-resilient, exhibit a lower level of anxiety, and have less emotionality than do BALB/c mice, which are generally more stress-sensitive, exhibit a high level of anxiety, and have increased emotionality.[2,6,30,36] In addition, evaluation of corticotrophin-releasing hormone receptor immunoreactivity indicates that BALB/c mice, which are more responsive to acute stress, are even more responsive to chronic stressors, whereas C57BL/6 mice, which are less responsive to acute stressors, acclimate to chronic stress more readily.[1] BALB/c mice typically are poorer learners and

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[1]Medical University of South Carolina, [2]Ralph H Johnson VA Medical Center Charleston, SC
**Corresponding author. Email: laberlk@musc.edu*

have poorer memory than do C57BL/6 mice.[6,28,33] In addition, prior studies[15,20,21,23] have demonstrated differences between the immunologic profiles of C57BL/6 and BALB/c strains. In particular, stress impairs T-cell responses to a greater extent in BALB/c mice as compared with C57BL/6 mice,[23] and BALB/c mice are more susceptible to various pathogens.[15,20,21]

Recent studies conducted using less floor space than recommended by the *Guide* found no deleterious effects on the mice.[31,32] Additional studies suggested that housing according to *Guide*-recommended spaces increased anxiety-like behavior.[9] In contrast, other studies have shown that increased housing densities increase aggressive behavior and plasma corticosterone levels.[4] However, interpretation of this finding was confounded by the additional finding that decreasing floor space in combination with increasing group size decreased aggressive behavior.[34] Many previous studies simultaneously manipulated both group size and cage size, thereby complicating elucidation of the effect of caging density alone. Furthermore, the ability to draw overarching recommendations is complicated by the use of multiple strains of mice. None of the cited studies provide in-depth analyses of the effects of housing density on immune parameters in combination with assessments of stress and anxiety-related behavior, nor have the studies evaluated whether the physiologic effect of a particular housing density varies over time. This issue becomes scientifically relevant if animals are housed at different housing densities at commercial vendor sites versus institutional sites and might affect the time required for animals to become physiologically stable after they have arrived at the institution.

The objective of the present study was to determine the effects of housing density on behavior, immune parameters, and plasma corticosterone levels in two frequently used strains of laboratory mice, one stress-sensitive (BALB/c) and the other stress-resilient (C57BL/6), housed over a 10 wk time period at 3 housing densities in standard shoebox-type ventilated caging, with the goal of providing additional scientific basis for rodent housing density recommendations.

Materials and Methods

Animals. Mature female C57BL/6 and BALB/c mice (Charles River Laboratories, Raleigh, NC) with initial weights of 22 to 24 g were used in the study. Female mice were chosen to avoid the potential for aggressive behavior, which can be an uncontrolled variable in group-housed male mice, and to accommodate comparison with our previously obtained immunologic data. Mice were allowed a 1-wk acclimation period at a housing density of 5 mice/cage. At the end of the acclimation period, they were weighed, ear-punched, and randomly assigned to a housing density of 2, 5, or 10 mice/cage. Mice were kept in a specific pathogen-free animal facility where quarterly sentinel surveillance was conducted. Sentinel mice were negative for *Mycoplasma pulmonis,* Sendai virus, mouse hepatitis virus, pneumonia virus of mice, reovirus 3, Theiler virus, *Ectromelia*, mouse adenovirus, polyoma virus, lymphocytotic choriomeningitis virus, cytomegalovirus, murine rotavirus, murine parvovirus, cilia-associated respiratory bacillus, and ecto- and endoparasites. Trained animal care staff provided daily health monitoring for the mice and weighed them weekly throughout the studies.

Caging and husbandry. The caging system comprised an individually ventilated caging rack (Lab Products, Seaford, DE) with cages (Supermouse Zytem, Lab Products) having interior dimensions of 12.88 × 7.50 × 5.63 in. (32.46 × 19.05 × 14.17 cm) and total floor area of 75 in^2 (484 cm^2). Mice rested directly on autoclaved corncob bedding (Bed-O'Cobs, The Andersons, Maumee, OH), and cotton nestlets (Ancare, Bellmore, NY) were included for environmental enrichment. A 12:12-h light:dark cycle was provided (0600 to 1800), and temperature and humidity were continuously monitored and maintained within *Guide* standards. The mice received reverse-osmosis–treated water via water bottles and were provided ad libitum access to autoclaved, certified laboratory diet (Mouse Diet 8656, Harlan Teklad, Bartonville, IL). Mice were transferred to clean cages with clean bedding once every 2 wk. A single, assigned caretaker made the cage transfers throughout the study. The experimental protocol was approved by the Ralph H Johnson VA Institutional Animal Care and Use Committee prior to animal use and in accordance with the guidelines of the *Guide*.[27]

Experimental design. At 7, 28, and 70 d after transfer to housing at the randomly assigned densities, half of the mice at each housing density were weighed and euthanized by decapitation, and then blood, spleens, and lymph nodes (inguinal, axillary, and mesenteric) were collected (Figure 1). To eliminate the confounding variable of circadian rhythm, all mice were euthanized at 0700. The remaining mice in each group were weighed and then underwent behavioral testing designed to determine the effect of the experimental manipulation on spontaneous behavior.

Behavioral assessment. Assessment of spontaneous locomotor activity in a modified open-field environment was accomplished by using videotracking (Smart DT System, Panlab, Barcelona, Spain). Prior to the initiation of testing, parameters delineating interior and exterior portions of an 8.5 × 16 × 8 in. (21.6 × 40.6 × 20.3 cm) cage were defined in the videotracking system such that the exterior portion spanned 2 in. (5 cm) in from the wall. Individual mice were placed in the cage on corncob bedding, and spontaneous locomotion was videotaped for 5 min. On completion of the 5 min period, the mouse was removed, the cage was cleaned, and fresh corncob bedding was placed in the cage. By using the defined parameters, the videotracking system calculated the amounts of time spent in the exterior and interior areas of the open field as well as transitions between the 2 areas.

Corticosterone measures. Plasma corticosterone levels were determined by radioimmunoassay.[22,35] ^{3}H-corticosterone (Perkin–Elmer, Waltham, MA) was incubated with a polyclonal rabbit antibody against corticosterone (MP Biochemicals, Solom, OH), and this complex removed by using a charcoal–dextran solution. Free ^{3}H-corticosterone was measured by liquid scintillation spectrometry.[22,35] Samples were assayed in duplicate, along with a standard curve and internal quality controls.

Immune parameters. The phenotype of splenic T-cell subpopulations was determined by immunostaining and flow cytometric analyses. T-cell subpopulations were identified by immunostaining for surface marker expression by using the conjugated antibodies PerCp-CD3, PE-CD8, and APC-CD4 (BD PharMingen, San Diego, CA) and enumeration by flow cytometric analyses (FACS Canto, BD Bioscience, San Diego, CA). $CD3^+$ cells were indicative of the total number of T-cells. $CD3^+CD4^+$ cells were indicative of helper T cells, whereas $CD3^+CD8^+$ cells were indicative of cytotoxic T cells. All analyses were conducted by using FACSDiva software (BD Bioscience).

Data analysis. Physiologic (change in body weight and levels of plasma corticosterone and immune cells) and behavioral (number of entries in central area and percentage time in the exterior area of an open field) data were analyzed separately by using general linear analysis of variance (GLM, SAS Institute, Cary, NC) with strain (BALB/c versus C57BL/6), housing den-

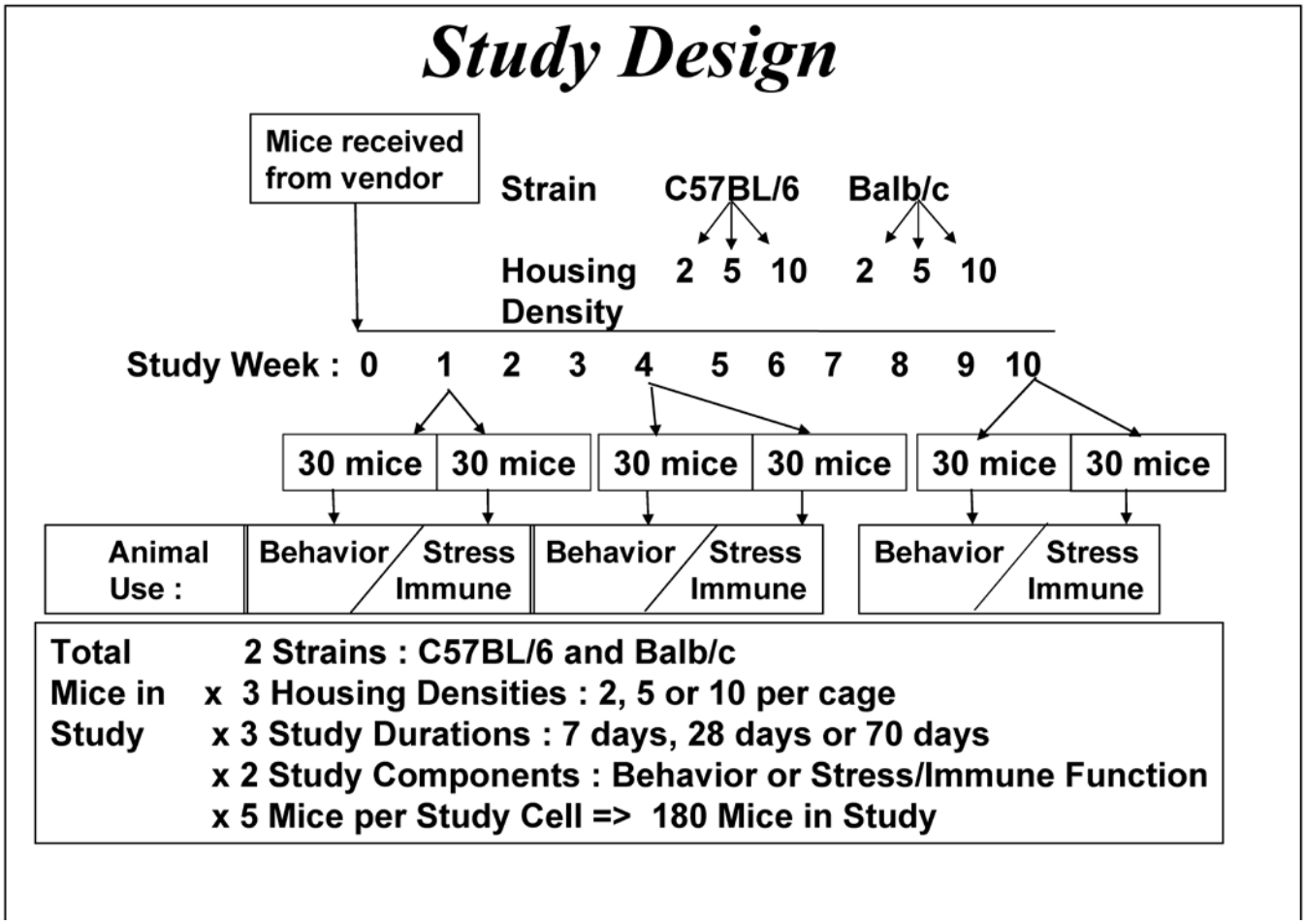

Figure 1. Experimental design. Each time point represents a single mouse that was used for terminal collection of blood (immune and stress assays) or behavioral testing.

sity (2 versus 5 versus 10) and duration of housing (7 d versus 28 d versus 70 d) serving as between-subject variables. Furthermore, due to the extensive literature documenting overall strain differences on these dependent variables between BALB/c and C57BL/6, an a priori decision was made to analyze data separately by strain. Throughout the analyses, statistical significance was set at $P < 0.05$, and post-hoc comparisons (least square means tests)(GLM, SAS Institute, Cary, NC) were conducted as appropriate. In addition, to begin investigation into potential relationship between physiologic and behavioral measures, these variables were entered into correlation analyses.

Results

Weight gain. The effect of housing density on weight gain in the 2 strains of mice at 7, 28, and 70 d are shown in Figure 2. As expected due to strain phenotype, C57BL/6 mice gained more weight during the course of the study than did BALB/c mice. Housing density did not statistically affect weight gain in the C57BL/6 mice at any of the time points measured. However, housing density altered weight gain by BALB/c mice at 7 d ($F[2, 27] = 10.7$, $P < 0.0004$), 28 d ($F[2, 27] = 5.9$, $P < 0.0075$), and 70 d ($F[2, 27] = 7.50$, $P < 0.0026$). At all 3 time points, BALB/c mice in high-density housing (10 mice/cage) gained less weight than did BALB/c mice housed at low densities (2 mice/cage). Weight gain by BALB/c mice housed at 5 mice/cage fluctuated between those associated with the low- and high-density housing conditions.

Behavior. Figure 3 depicts the number of entries into the central area of the open field by BALB/c (Figure 3 A) and C57BL/6 (Figure 3 B) mice housed at 2, 5, or 10 per cage for 7, 28, or 70 d. Overall statistical analysis evaluating the effects of strain, housing density, and housing duration on this measure of exploratory behavior indicated significant main effects of strain ($F[1,72] = 92.19$, $P < 0.0001$), housing density ($F[2,72] = 9.11$, $P < 0.0003$), housing duration ($F[2,72] = 5.5$, $P < 0.0060$), and a significant interaction of strain × housing duration ($F[2,72] = 3.93$, $P < 0.0240$). Post-hoc analyses of these main effects revealed that 1) mice housed in low-density (2/cage) conditions showed more exploratory behavior than did those housed at medium (5/cage) or high (10/cage) density; 2) mice housed for 70 d showed more exploratory behavior than did those housed for 7 or 28 d; and 3) overall exploratory behavior was higher in C57BL/6 mice than BALB/c mice.

Given the significant main effect of strain, we performed individual analyses by strain to evaluate the effect of housing density and duration on exploratory behavior. At 70 d in both strains, mice housed in high-density conditions (10/cage) demonstrated significantly lower levels of exploratory behavior than did mice housed in low-density conditions (BALB/c: $F[2,14] = 6.57$, $P < 0.0118$; C57BL/6: $F[2,14] = 5.43$, $P < 0.0209$). Exploratory behavior of BALB/c mice significantly ($P < 0.05$) increased over time in an inverse relation to the housing density; this did not occur in the C57BL/6 mice. Therefore, after 70 d of housing, the exploratory behavior of BALB/c mice in the low

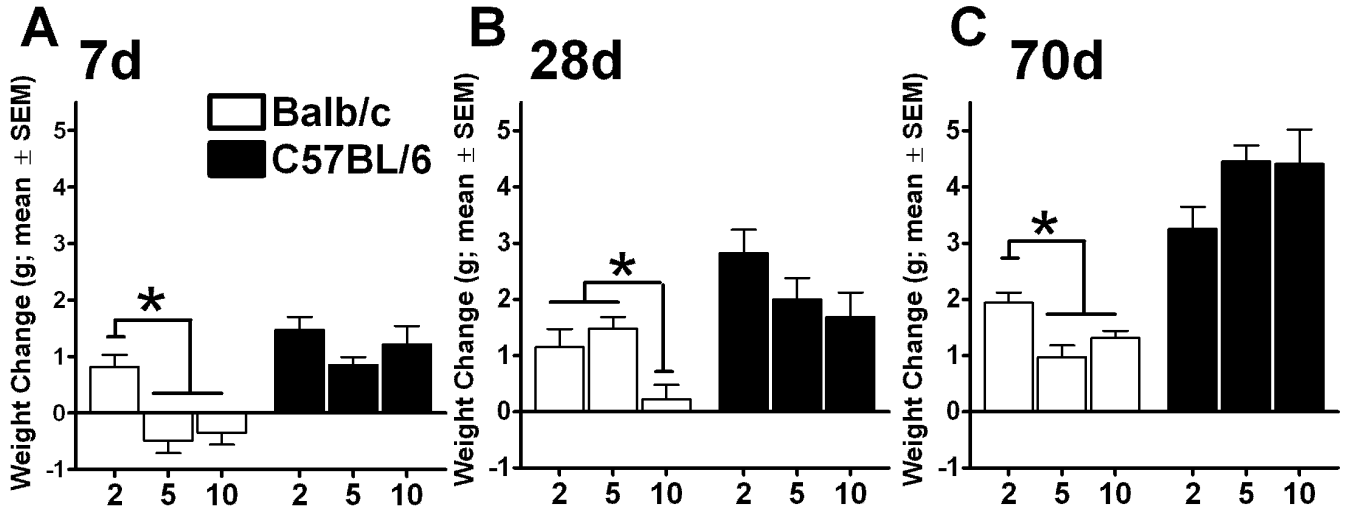

Figure 2. Weight change (grams of weight gained from time 0) in BALB/c and C57BL/6 mice housed at 2, 5, or 10 mice/cage for (A) 7 d, (B) 28 d, or (C) 70 d. Throughout the experiment, BALB/c mice housed at 2/cage gained significantly more weight than did BALB/c mice housed at 10/cage. This effect was not seen in C57BL/6 mice. *, $P < 0.05$ (3-way ANOVA).

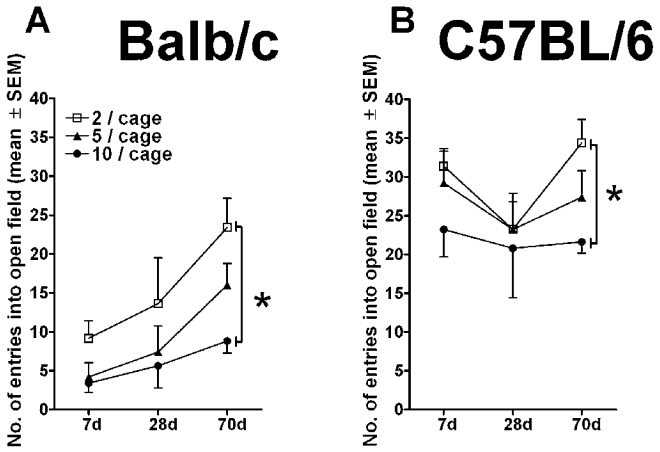

Figure 3. The number of entries into a central area of the open field by (A) BALB/c and (B) C57BL/6 mice housed at 2, 5 or 10 mice/cage for 7, 28, or 70 d. In both strains, mice housed at lower densities generally had more open field entries than did those housed at higher densities. *, $P < 0.05$ (3-way ANOVA).

density (2/cage) condition was similar to that of C57BL/6 mice housed at all densities.

Figure 4 depicts the percentage of time spent in the outer portion of the open field by BALB/c and C57BL/6 mice housed at 2, 5, or 10/cage for 7 d (Figure 3 A), 28 d (Figure 3 B), or 70 d (Figure 3 C). Overall statistical analysis evaluating the impact of strain, housing density, and housing duration on this measure of exploratory behavior showed a significant main effect of strain alone ($F[1,72] = 6.38$, $P < 0.0138$). Statistical analyses indicated that the BALB/c mice were significantly affected by housing duration ($F[2, 36] = 4.83$, $P < 0.0139$), with housing density showing a trend ($F[2,36] = 3.04$, $P < 0.0605$) and no significant interaction. Further analysis of the housing duration main effect in BALB/c mice demonstrated that, after 70 d, BALB/c mice housed in high-density (10/cage) conditions spent more time in the outer area of the open field than did those housed at low (2/cage) or medium (5/cage) densities. In contrast, the time spent in the outer field by the C57BL/6 mice was not significantly affected by housing density and duration.

Corticosterone. Figure 5 depicts plasma corticosterone levels expressed by BALB/c and C57BL/6 mice housed for 7 d (Figure 5 A), 28 d (Figure 5 B), or 70 d (Figure 5 C) at low (2/cage), medium (5/cage), or high (10/cage) housing density. Overall statistical analysis evaluating the impact of strain, housing density and housing duration on plasma corticosterone levels indicated a significant main effect of housing density ($F[2,72] = 16.24$, $P < 0.0001$). Post-hoc analysis indicated that corticosterone levels were significantly and systematically elevated by increasing housing density. In C57BL/6 mice, housing density was a significant factor affecting corticosterone levels ($F[2,36] = 6.18$, $P < 0.0049$), with mice housed in low (2/cage) density conditions having lower corticosterone levels. However, the medium- (5/cage) and high- (10/cage) housed C57Bl/6 mice did not show statistically relevant differences for the measured time points. In contrast, the effects of housing density and housing duration on plasma corticosterone levels in BALB/c mice were consistent and systematic. Similar to findings from the analysis of C57BL/6 data, overall analysis of BALB/c corticosterone levels indicated a significant main effect of housing density ($F[2,36] = 12.12$, $P < 0.0001$), with mice in the high- (10/cage) density condition exhibiting higher corticosterone levels than those housed at low and medium densities. In contrast to C57BL/6 data, BALB/c mice tested at 7 d showed a trend ($P = 0.09$) for corticosterone levels to differ by housing density condition. At both 28 and 70 d, this effect was significant in the BALB/c mice ($F[2, 12] = 9.86$, $P < 0.0029$, and $F[2, 12] = 4.45$, $P < 0.0358$, respectively). Post-hoc analyses indicated that at the 28- and 70-d time points, corticosterone levels in BALB/c mice in the high-density (10/cage) condition were higher than levels in the BALB/c mice at the low-density (2/cage) condition. At 28 d, corticosterone levels in the BALB/c mice in the medium-density (5/cage) condition were similar to those at the low-density (2/cage) condition, however by 70 d, they were more similar to levels of the high-density (10/cage) condition.

Immune parameters. Figure 6 depicts the levels of total T cells ($CD3^+$; Figure 6 A), and further identification of $CD4^+$ helper T cells (Figure 6 B) and $CD8^+$ cytotoxic T cells (Figure 6 C) in BALB/c and C57BL/6 mice housed at low (2/cage), medium (5/cage), or high (10/cage) density for 70 d. Whereas overall levels of total T cells ($CD3^+$) did not differ between strains or among housing densities, $CD4^+$ helper T-cell and $CD8^+$ cytotoxic

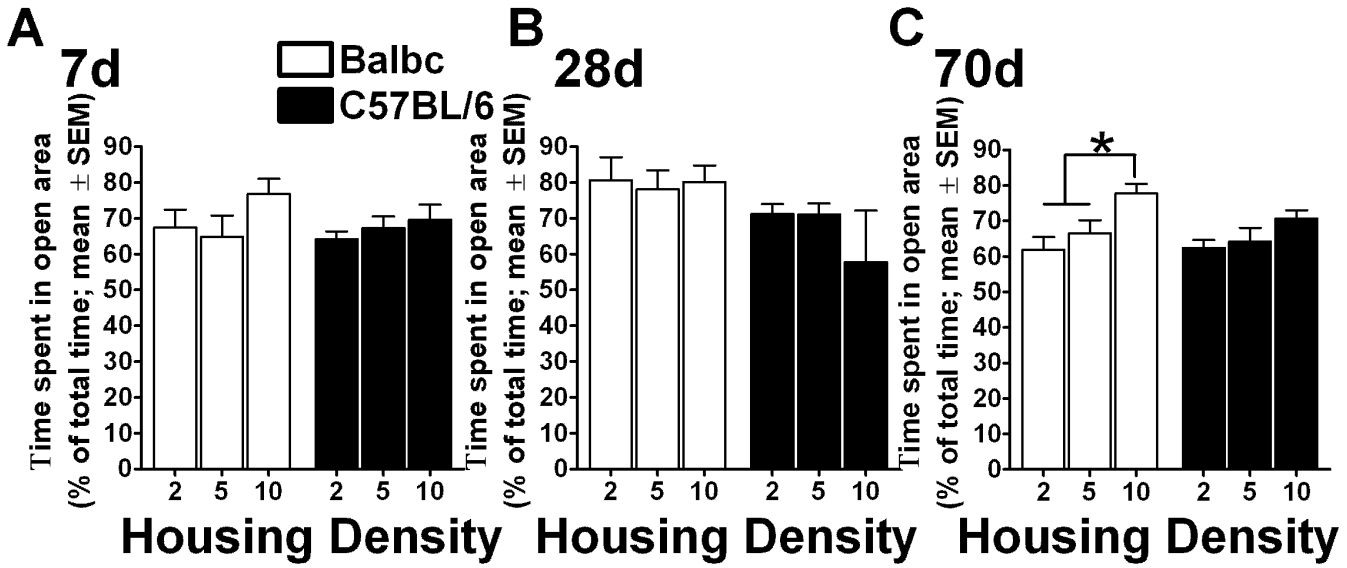

Figure 4. The percentage of time spent in the outer wall area by BALB/c and C57BL/6 mice housed at 2, 5, or 10 mice/cage for (A) 7 d, (B) 28 d, or (C) 70 d. After 70 d, BALB/c mice housed at 10/cage spent more time in the outer wall area than did BALB/c housed at either 2/cage or 5/cage. Housing density did not affect this measure in C57BL/6 mice. *, $P < 0.05$ (3-way ANOVA).

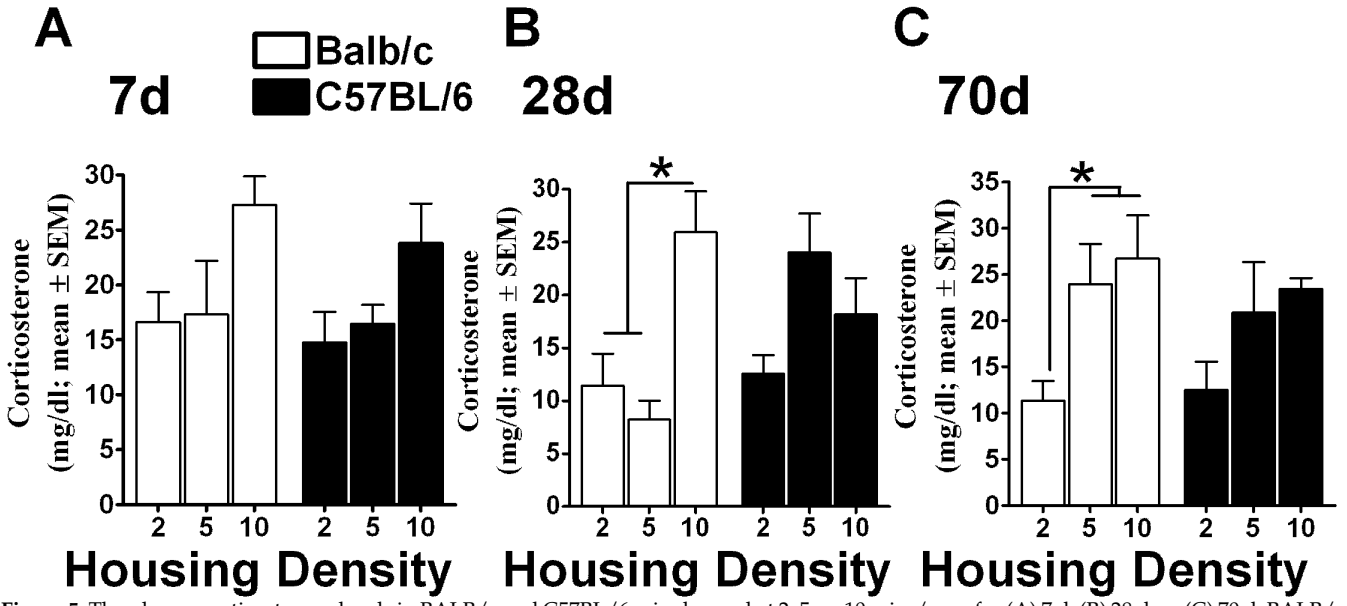

Figure 5. The plasma corticosterone levels in BALB/c and C57BL/6 mice housed at 2, 5, or 10 mice/cage for (A) 7 d, (B) 28 d, or (C) 70 d. BALB/c mice housed at high density (10/cage) had higher corticosterone levels than did those housed at low density (2/cage). Mice housed at medium density (5/cage) initially mirrored the low-density group. However, by the 70-d time point, corticosterone levels for the mice housed at medium density became more similar to those of mice housed at high density. This effect was not seen in C57BL/6 mice. *, $P < 0.05$ (3-way ANOVA).

T-cell subpopulations were affected by both strain and housing density. Statistical analysis of helper T-cell ($CD4^+$) levels showed a significant main effect of strain ($F[1, 24] = 40.15$, $P < 0.0001$), with levels in BALB/c mice higher than those of C57BL/6 mice. Given the significant strain main effect, we performed separate analyses evaluating the effect of housing density on each strain. The results indicated that $CD4^+$ levels were lower in BALB/c mice in the high-density (10/cage) condition compared with BALB/c mice housed in the medium- (5/cage) and low- (2/cage) density conditions. In contrast, $CD4^+$ levels in C57BL/6 mice were not affected by differences in housing density. In addition, statistical analysis of cytotoxic T-cell ($CD8^+$) levels indicated a significant main effect of strain ($F[1,24] = 20.16$, $P < 0.0002$), with $CD8^+$ levels in C57BL/6 mice higher than those in BALB/c mice. Further analysis by strain indicated no effect of housing density on this measure.

Relationship between corticosterone and immune response. Exploratory analyses of the relation between plasma corticosterone and levels of $CD4^+$ were performed. Figure 7 indicates a significant negative correlation ($P < 0.0451$) between plasma corticosterone and $CD4^+$ levels in the BALB/c mice housed for 70 d. Furthermore, plotting individual data points as corticosterone by housing density, a cluster of BALB/c mice in the low-density (2/cage) condition demonstrate lower corticosterone and higher $CD4^+$ levels, whereas BALB/c mice in the high-density (10/cage) condition demonstrate the reverse pattern (higher corticosterone and lower $CD4^+$ levels).

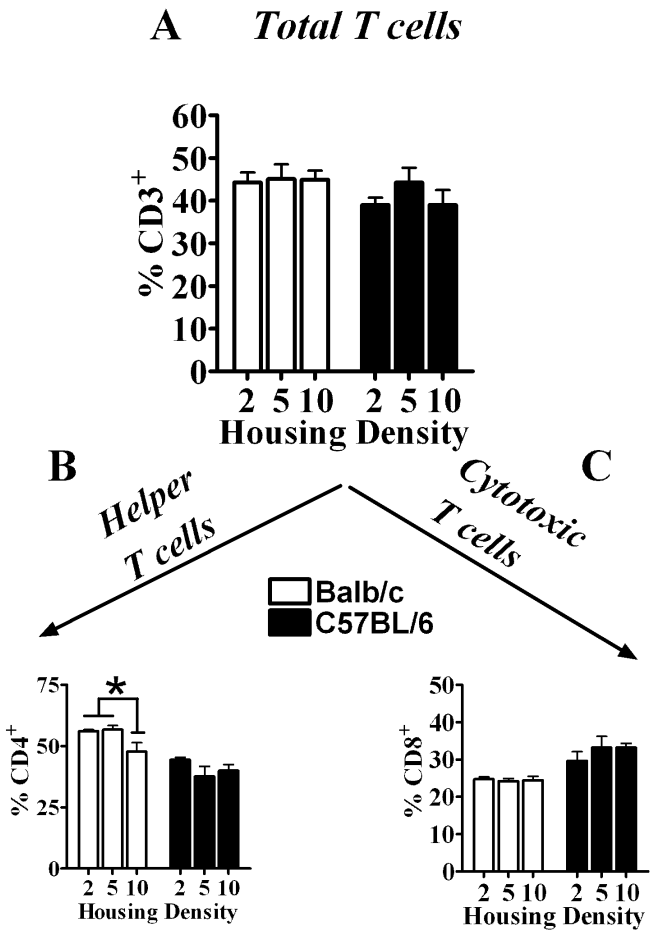

Figure 6. Percentage of splenic (A) total, (B) helper, and (C) cytotoxic T cells in BALB/c and C57BL/6 mice housed at 2, 5 or 10 mice/cage at the 70-d time point. Percentage of total T cells was unaffected by housing density in both strains (A). However, BALB/c mice housed at 10/cage had significantly lower levels of helper T cells than did those housed at the lower densities (B). Housing density had no effect on cytotoxic T cell levels in BALB/c mice (C) or on any T cell population in C57BL/6 mice. *, $P < 0.05$.

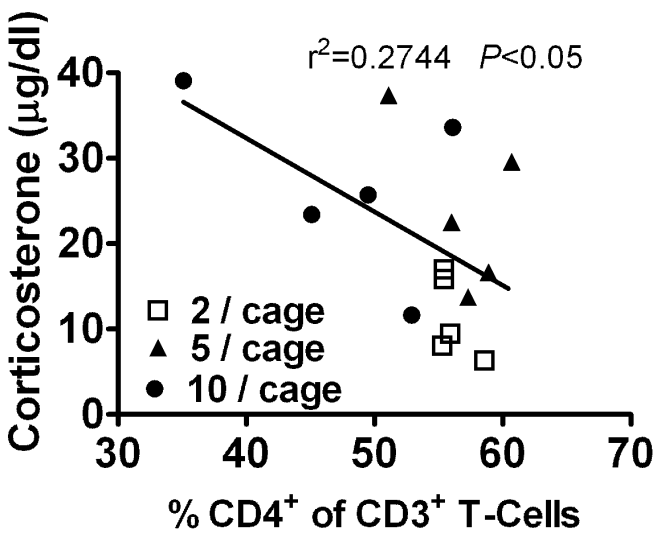

Figure 7. Correlation of number of helper T cells to corticosterone levels in BALB/c mice housed at 2, 5 or 10 mice/cage for 70 d. An inverse relationship was present, with low helper T-cell levels correlating with high corticosterone levels. Furthermore, a clustering of housing densities was present. *, $P < 0.05$).

Discussion

The knowledge that various environmental variables affect the physiology of mice used in biomedical research has led to standardization of many of these variables such that scientific results can be duplicated and validated.[19] Housing density is another environmental variable that could alter mouse physiology and subsequently affect scientific studies, particularly those in which behavioral analysis and immune function are involved. The results of our study demonstrate that housing density does affect mouse physiology and behavior and that the effect is strain-dependent. Therefore, rodent housing density standards should be clearly defined and maintained to allow research to be conducted in a reproducible manner.

High-housing density had a greater effect on BALB/c mice throughout the study. At all 3 time points, the BALB/c mice that were housed at high density (10/cage) gained less weight than did BALB/c mice housed at low density (2/cage). The study also indicated that behavioral and chemical effects of housing increased over time. The BALB/c mice housed at 10/cage had higher corticosterone levels, spent more time in the outer portion of the open field, and had fewer entries into the open-field area; these quantifiable physiologic measures and behavioral parameters have been related to anxiety or stress.[12] Because neophobia is a prominent feature of BALB/c behavior,[2] we anticipated that they would have less exploratory behavior than C57BL/6 mice. We also anticipated that variation in housing density would cause a more marked response in BALB/c mice.

The effect of housing density was less robust but still apparent in C57BL/6 mice. Although weight gain was not affected, housing density did affect some of the measures of behavior. At 70 d, C57BL/6 mice housed in the high-density condition (10/cage) showed less exploratory behavior compared with those of their counterparts housed at low density (2/cage), and although not achieving statistical significance, this trend was evident both at the 7- and 28-d time points. Although housing density affected our measure of exploratory behavior in C57Bl/6 mice, it did not alter the percentage of time spent in the outer wall of the open field, reflecting hesitancy and uncertainty. These findings support previous reports[13,25] that C57B/6 mice are relatively resilient in the face of environmental stressors that would provoke signs of fear and anxiety in other strains of mice.

We recognize that the locomotion is only one component of a wide array of open-field behaviors that could be used to determine the effect of housing density on behavior of mice and that exploratory behavior, which we extrapolated from number of entries into the central field, is influenced both by the genetically programmed need to explore balanced against the need to guard against predation.[7,25] The differences we saw in behavioral measures may largely have been predictable based on the genetics of the strain. However the change in our measures of behavior over time helps to validate that housing density did have an effect independent of genetic predisposition.

Overall levels of total T cells did not differ betweens strains or among housing densities, however helper T-cell ($CD4^+$) subpopulations were affected by strain and housing density. BALB/c mice overall had higher levels of helper T cells ($CD4^+$) than did C57BL/6 mice. However, helper T-cell ($CD4^+$) levels were lower in BALB/c mice housed at high density (10/cage) as compared with those of mice at lower housing densities. Housing conditions did not affect immune cells populations in C57BL/6 mice. Our results are consistent with prior studies demonstrating that T-cell responses were diminished after stress in BALB/c mice as compared with C57BL/6 mice.[23] These

earlier findings demonstrating changes in T-cell function are extended by our results, because we show that the $CD4^+$ T cells are the affected subpopulation.

The inverse relationship between corticosterone and $CD4^+$ T-cell levels in BALB/c mice in our study further supports an effect on T-cell subpopulations. Prior studies have established that cytokine secretion by immune cells is modulated in part by glucocorticoids.[11] Given the significant correlation between corticosterone and $CD4^+$ cells in BALB/c mice, we postulate that persistent corticosterone elevation over time affects the secretion profile of cytokines by macrophages, subsequently altering $CD4^+$ differentiation. Analysis to further delineate the effect of housing density on immune parameters is underway, as are studies to assess the correlations between specific immune parameters and the other dependent measures determined in this study.

Previous studies of similar design (that is, manipulation of the number of mice housed in a cage, rather than cage size) indicated that housing density did not affect weight in C57BL/6 mice.[9,14] Our findings are consistent with that finding for this specific strain of mice. However, in BALB/c mice, high cage density significantly reduced weight gain, a finding that conflicts with previously reported data.[24] The previous studies for C57BL/6[9,14] and BALB/c mice[24] also extrapolated that *Guide*-recommended cage densities had a negative impact on immune function; this interpretation was confounded in C57BL/6 mice by the finding of higher plasma glucocorticoid levels. Although a trend toward higher corticosterone levels at higher housing densities was present in C57BL/6 mice, our data indicate that housing density significantly affected corticosterone levels and immune function only in BALB/c mice and that housing according to *Guide* recommendations did not perturb immune function. The differences between studies could be attributable in part to the different genders of mice used.

The scope of this study was limited in that it encompassed only 2 strains of female mice and 4 dependent variables. Furthermore, overarching conclusions regarding housing densities in C57Bl/6 and BALB/c mice are not possible because the study used only female mice. A previous study[25] showed both gender and strain-associated differences in behavioral responses to stress. Therefore, further studies using male mice are necessary to support broader conclusions regarding C57Bl/6 and BALB/c mice.

Even if male mice had been included in this study, the effect of housing density on all potential variables for all strains of mice could not be assessed unequivocally. The question becomes even more complex when environmental enrichment is added to cages and when group compositions are varied. The present study supports the conclusion that the optimal housing density for mice varies with the strain used and the dependent measures evaluated. Therefore, promulgation of a universal, scientifically based best practice for housing density of all mouse strains used in research is likely impossible. Nonetheless, operating under the premise that solid scientific design requires the control of variables that affect research and that housing density is such a variable, the ability to define a standard for the housing density of mice becomes imperative.

Of note, the effect of housing BALB/c mice at 5/cage vacillates between being statistically different from mice housed at 2/cage and those housed at 10/cage for both weight gain and corticosterone levels at the time points measured. A similar albeit nonsignificant trend also occurred in C57BL/6 mice, lending support to the concept that animals housed at 5/cage are physiologically balanced between those housed at 2/cage and 10/cage. These data support the view that the professional judgment and experience reflected in the *Guide* standards for rodent housing have stood the test of time and science, at least for the strains and variables that were assessed in this study. Although animal welfare must always be a critical factor in designing housing for animals maintained in research environments, the practical implications of housing systems relevant to the conduct of science also must be considered. Many institutions that contribute to the base of scientific knowledge are supported by NIH and therefore are required to house mice at the recommended *Guide* densities.[26] Forty years of scientific data have been produced using these cage densities. Unless an alternative cage density or cage space can be unequivocally documented to significantly improve animal welfare, the cost to science of redefining the current cage density standard appears untenable.

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