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The Calm Mouse Model of Stress Reduction

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The Calm Mouse: An Animal Model of Stress Reduction

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Chronic stress is associated with negative health outcomes and is linked with neuroendocrine changes, deleterious effects on innate and adaptive immunity, and central nervous system neuropathology. Although stress management is commonly advocated clinically, there is insufficient mechanistic understanding of how decreasing stress affects disease pathogenesis. Therefore, we have developed a "calm mouse model" with caging enhancements designed to reduce murine stress. Male BALB/c mice were divided into four groups: control (Cntl), standard caging; calm (Calm), large caging to reduce animal density, a cardboard nest box for shelter, paper nesting material to promote innate nesting behavior, and a polycarbonate tube to mimic tunneling; control exercise (Cntl Ex), standard caging with a running wheel, known to reduce stress; and calm exercise (Calm Ex), calm caging with a running wheel. Calm, Cntl Ex and Calm Ex animals exhibited significantly less corticosterone production than Cntl animals. We also observed changes in spleen mass, and *in vitro* splenocyte studies demonstrated that Calm Ex animals had innate and adaptive immune responses that were more sensitive to acute handling stress than those in Cntl. Calm animals gained greater body mass than Cntl, although they had similar food intake, and we also observed changes in body composition, using magnetic resonance imaging. Together, our results suggest that the Calm mouse model represents a promising approach to studying the biological effects of stress reduction in the context of health and in conjunction with existing disease models.

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INTRODUCTION

Environmental stress is a pervasive dimension of life that results in complex biological changes. Evidence suggests that chronic stress can exert negative effects on general health, disease susceptibility, and progression of existing illness (1). Networks that are stimulated by stress include: the hypothalamic–pituitary–adrenal (HPA) axis, the sympathetic adrenal medullary axis and sympathetic and parasympathetic nerve projections that directly innervate secondary lymphoid organs (2–4). Repeated or prolonged exposure to stress-related neuroendocrine factors, such as glucocorticoids and catecholamines, can potently influence immune function and is believed to underlie the adverse health outcomes associated with chronic stress (2,3). For example, human studies have shown that chronically stressed individuals exhibit poorer influenza vaccine responses (5,6), enhanced susceptibility to experimental rhinovirus infection (7) and accelerated human immunodeficiency virus disease progression (8). Research employing animal models has demonstrated that chronic stress impairs innate and adaptive immunity (9–11) and can yield enhanced tumor growth (12,13) or increased simian immunodeficiency virus–associated mortality (14). Other work has also linked stress and glucocorticoid production with hippocampal atrophy and memory dysfunction in both humans and animals (15–17).

To mitigate the negative physiologic effects of stress, stress management approaches are often advocated by health professionals. Stress reduction tech-

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niques, such as mindfulness-based stress reduction, have been shown to reduce anxiety and depression, influence the HPA axis and may affect immunity (18–22). Although clinical studies of stress management are informative, they do not identify the mechanistic pathways that are modulated in response to reduced stress exposure, leaving uncertainty about how stress reduction influences biology and which pathways are important to influence for specific health outcomes. Furthermore, prior animal studies have focused primarily on increasing stress, and few animal models of stress reduction have been fully developed.

Here, we describe a "calm mouse model" of stress reduction in which we have altered several facets of the mouse-caging environment. Environmental enrichment protocols have been shown to reduce the negative effects of both psychogenic and neurogenic stress in rodent models (for review see 23). Studies probing the stress-related effects of environmental enrichment have demonstrated that animals housed in enriched caging exhibit enhanced stress resiliency (24) and that reduced expression of amygdalar corticotropin releasing factor receptor type 1 is linked to the anxiolytic effects of an enriched environment (25). Interestingly, published studies in which environmental enrichment protocols were used have revealed both increased (26–28) and decreased (25,29) corticosterone production, a physiologic indicator of stress. We hypothesized that the increased corticosterone levels found in some of these studies may have been due to excessive cage complexity or frequent rotation of enrichment items within a cage. Thus, we omitted those elements from our design and carefully selected caging enhancements with the intention of minimizing stress levels. For example, increasing the amount of floor space per animal within a cage is associated with reduced glucocorticoid production and adrenal gland mass (30). Other work has shown that mice prefer cages that are equipped with enhanced nesting material and that shredded paper strips are most similar to materials that mice use to build three-dimensional nests in natural settings (31). Flat-roofed cardboard nest boxes and polycarbonate tubes provide auxiliary hiding spaces and increase cage complexity, characteristics that mice prefer (32). Lastly, in humans, cardiovascular exercise is associated with stress reduction (33), and animal studies have shown that mice will carry out tasks, like bar pressing, to engage in voluntary wheel running, which suggests that access to a running wheel is a rewarding enhancement (34).

In this study, male BALB/c mice were organized into four arms: control (Cntl), standard caging; calm (Calm), large caging, a cardboard nest box, paper nesting material, and a polycarbonate tube; control exercise (Cntl Ex), standard caging with a running wheel; and calm exercise (Calm Ex), Calm caging with a running wheel. We examined physiological, biological and behavioral parameters in mice housed in each condition to investigate whether Calm caging, access to an exercise wheel, or the combination of both caging enhancements could effectively reduce stress levels in mice. Findings from this study indicate that the Calm mouse model achieved reductions in physiologic stress measures and is a promising approach to modeling the biological effects of stress reduction.

MATERIALS AND METHODS

Animals

Forty 8-wk-old male BALB/c mice were purchased from Charles River (Hollister, CA, USA) and housed in the Laboratory Animal Resource Center (LARC) at San Francisco General Hospital. Cages were kept in a temperature-controlled room (22°C) with a light–dark 12:12 cycle (light on 0600–1800 h). All studies were approved by the University of California San Francisco Institutional Animal Care and Use Committee and were conducted in accordance with national guidelines of humane laboratory animal care.

Diet

All cages were provided with water and PicoLab Rodent Diet 20 (LabDiet, PMI Nutrition International, St. Louis, MO, USA) *ad libitum*. Percent calories provided by macronutrients were: protein, 24.651%; fat, 13.205%; carbohydrates, 62.144%; and crude fiber contents: not more than 6.0%.

Timeline

After arrival, all mice were group housed (five animals per cage) in standard control caging for a period of 14 d (d –14 to 0) to allow them to acclimatize to the LARC husbandry environment. After acclimatization, animals were placed in their respective caging conditions and remained there for an additional 70 d. The elevated plus maze (EPM) was carried out at d 42. Animals were weighed during weekly cage cleaning and measures of dry food consumption were recorded by cage. Additionally, fecal samples were taken from each animal at regular intervals and kept at –20°C for batched fecal corticosterone analysis (see below).

Caging and Enrichment

Mice were randomly assigned to four groups (n = 10 per group): Cntl, Calm, Cntl Ex or Calm Ex. For all groups, each cage housed five animals (two cages per group). All caging, bedding and enrichment items were autoclaved before use or sterilized with Coverage disinfectant spray (Steris, Mentor, OH, USA). Standard wire-bar lids for food and water and filter-top bonnets were used for all cages. Cages for the Cntl group (189 mm × 297 mm × 128 mm; 484 cm^2 surface area; Allentown Inc., Allentown, NJ, USA) contained standard Paperchip bedding (Shepherd Specialty Papers, Milford, NJ, USA) and lacked enhancements. Cages for the Calm group were larger (257 mm × 483 mm × 152 mm; 980 cm^2 surface area) and contained standard bedding, 1 paper nest box (Bio-Serv, Frenchtown, NJ, USA), 1 red polycarbonate mouse tunnel (Bio-Serv) and 236 cm^3 (1 cup) of compressed Enviro-

dri Eco-bedding shredded paper strips (FiberCore, LLC, Clevland, OH, USA). Cages for the Cntl Ex group contained the same amount of surface area as Cntl cages (312 × 235 × 152 mm; 484 cm^2 surface area), standard bedding and a green Silent Spinner running wheel (Super Pet, Elk Grove Village, IL, USA). Cages for the Calm Ex group were the same size (980 cm^2 surface area) and contained the same enhancements as Calm caging with the addition of a green Silent Spinner running wheel (Super Pet). Once weekly, cages and washable enrichment items were cleaned, and bedding, Enviro-dri strips and nest boxes were replaced. Note: Green running wheels were used in these experiments; however, identical running wheels in red color were used for display in Figures 1D, G, H. To fit the running wheel in Cntl Ex caging, the metal stand was removed and the plastic wheel mount was sawed and sanded flat to facilitate adhering of the wheel mount to the inner cage wall. The wheel mount was scored with a utility knife and bonded to the cage wall by use of water resistant epoxy glue and left to dry for 10 d before use.

Behavior

At d 42 (08:00), each animal was placed on an EPM and behavior was recorded via digital video camera for 5 min (Stoelting Co., Wood Dale, IL, USA). The maze was elevated to a height of 40 cm with 2 open arms (5 × 35 cm) and 2 closed arms (5 × 35 × 15 cm). Lighting conditions were kept low, with the center platform of the maze measuring <200 lux. At the beginning of each experiment the mouse was placed on the center platform facing an open arm and after each run the platform was thoroughly cleaned with disinfectant. Behavior analysis was carried out by two blinded observers and the results were expressed as: percent time spent in the open arms (open-arm time/[open-arm time + closed-arm time]), percent time spent in the closed arms. Placement of all four limbs within a maze arm or the center platform constituted an entry. Distance traveled was measured using semi-automated object tracking in ImageJ v1.43n software (ImageJ; National Institutes of Health [NIH], Bethesda, MD, USA). Data were expressed in distance traveled as percent of Cntl.

Body Composition

We determined body composition using magnetic resonance imaging (MRI). At the experimental endpoint, animals were killed and then frozen at –20°C until imaging. Animals were fully thawed overnight and imaged at 23°C. Imaging was performed with Agilent VnmrJ software v3.1 and a 7 Tesla (310-mm bore size) superconducting magnet equipped with actively shielded imaging gradients (400 mT/m maximum gradient strength, 120-mm inner bore size; Agilent Technologies, Palo Alto, CA, USA). A 72-mm inner diameter quadrature ^1H birdcage resonator (RAPID Biomedical GmbH, Rimpar, Germany) was used for radiofrequency pulse transmission and signal reception. Mice were positioned in the middle of the coil to achieve a complete radiofrequency field covering of the animal. The location of each animal was checked with a localizer sequence (gradient-echo, repetition time [TR] = 10 ms; echo time [TE] = 3.4 ms; field of view [FOV] = 120 × 120 mm^2; acquisition matrix = 512 × 512 mm^2, slice thickness = 2 mm; three orthogonal slices). A global iterative shim procedure was used to improve the field homogeneity. Lipid-sensitive imaging was performed by using a three-dimensional fast-spin–echo sequence with the following parameters: TR = 200 ms; effective echo time (TE_{eff}) = 45.1 ms; TE = 5.64 ms; echo train length = 16; FOV = 100 × 37.5 × 37.5 mm^3; acquisition matrix = 256 × 96 × 96 mm^3; resolution = 390 × 390 × 390 μm^3; receiver bandwidth = 156.2 kHz; number of averages = 4; number of dummy scans = 2; total acquisition time = 7 min 42 s. Image analysis was fully automatic and performed in Medical Imaging Processing, Analysis and Visualization (NIH). The fuzzy c-means algorithm was used to hard-segment the image into five tissue classes, the brightest of which was a reliable fat mask. This was converted into a volume of interest and volume measured and expressed as lipid volume per unit body mass (mm^3/g).

Fecal Corticosterone Metabolites

During cage changing, animals were temporarily placed in individual containers, and fecal pellets produced at this time were collected and stored at –20°C. Glucocorticoid metabolite quantification in fecal samples has been established in a large number of species and has been extensively validated for laboratory mice (35–37). Collected samples were analyzed for immunoreactive corticosterone metabolites by use of a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) as described previously (35,38). Briefly, fecal samples were homogenized and aliquots of 0.05 g were extracted with 1 mL of 80% methanol as described (35). The EIA used a double-antibody technique and was performed on anti-rabbit immunoglobin G–coated microtiter plates. After overnight incubation (4°C) of standards (range: 0.8–200 pg/well) and samples with steroid antibody and biotinylated label, the plates were emptied, washed and then 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 1 mol/L sulphuric acid. Then, the optical density (at 450 nm) was recorded with an automatic plate reader and the hormone concentrations were calculated. Samples were run in one batch to enhance data measure consistency. The intra- and interassay coefficients were 8.8% and 13.4%, respectively.

In Vitro Studies

At the experimental endpoint, d 70, animals were killed one at a time via cardiac puncture while under deep isoflu-

rane anesthesia. This process required approximately 10–15 min per cage. Depth and duration of anesthesia was similar for all animals. Sample collection was done within 2 h of lights on in the husbandry facility. Spleens were taken and cells were mechanically dissociated. Cell number was counted with standard methods after red blood cell lysis (eBioscience, San Diego, CA, USA). Purified splenocytes were plated at 2.5×10^5 cells per well in 200 μL of culture medium in a flat-bottom or round-bottom 96-well plate. Standard splenocyte culture medium was used with the addition of 5% charcoal-stripped fetal bovine serum (FBS), which contains minimal quantities of hormonal agonists of the glucocorticoid receptor. Cells were stimulated with LPS (1 μg/mL; Sigma Aldrich, St. Louis, MO, USA), CD3/CD28-coated Dynal beads (1 bead per cell; Invitrogen, Carlsbad, CA, USA), or vehicle control and treated with several concentrations of corticosterone (0.005 μmol/L; 0.025 μmol/L; 0.05 μmol/L; 0.075 μmol/L; Sigma Aldrich). All conditions were run in duplicate. At 18 h supernatants were collected and frozen at –80°C and later used to measure quantities of interleukin 6 (IL-6) and IL-2 via enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions (R&D, Minneapolis, MN, USA).

Serum Assays

At the experimental endpoint, whole blood was collected via cardiac puncture (see *In Vitro* Studies above), within 2 h of lights on, and supernatants were frozen at –20°C. ELISA kits were used as instructed by the manufacturer to measure serum levels of corticosterone (IBL America, Minneapolis, MN, USA), insulin (Millipore, Billerica, MA, USA) and insulin-like growth factor 1 (IGF-1) (R&D).

Statistical Analysis

Analyses were performed with Prism software v5.0a (GraphPad Inc., San Diego, CA, USA). Repeated measures analysis of variance (ANOVA) was used for body mass analysis with SAS PROC GLM v9.2 (Figure 2A). Unpaired *t* tests were used to compare experimental groups with Cntl for: body mass (Figures 2B–D), adiposity (Figure 2G), serum IGF-1 production (Figure 2H), corticosterone metabolite (CM) level (Figure 3), cytokine production (Figures 4B, C) and behavior (Figures 5A, B). Two-way ANOVA was used for measuring differences in energy intake at individual time points (statistical significance displayed for main effect of exercise, Figure 2E) and spleen mass (Figure 4A). Linear regression followed by two-tailed statistical comparison of regression coefficients was used to compare the relationship between serum insulin level and final body mass between groups (Figure 2J). Similarly, regression lines were used to illustrate the association between cytokine production and endpoint serum corticosterone level (Figures 4D, E). In all cases, $P < 0.05$ was considered statistically significant.

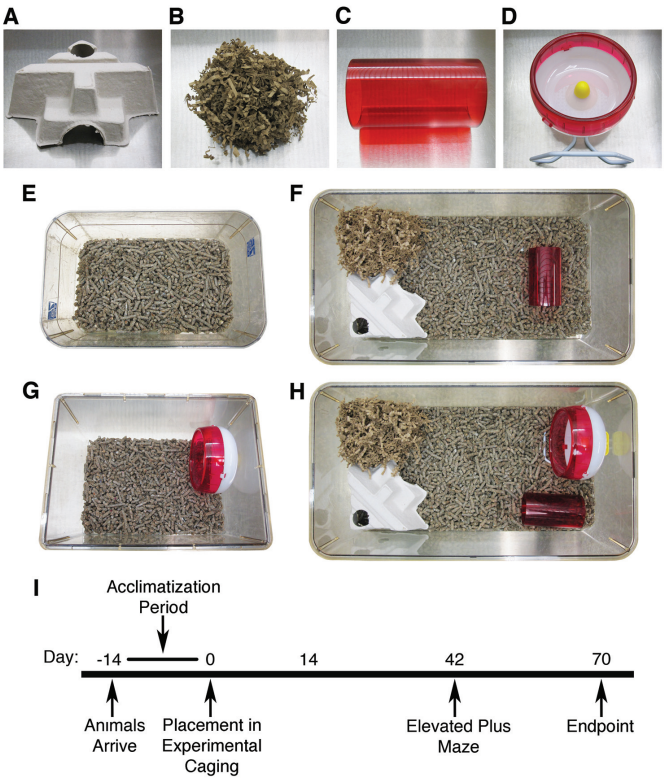

Figure 1. Enhanced caging and experimental design. Enrichment items were placed in experimental cages depending on condition and included a cardboard nest box (A), shredded paper nesting material (B), polycarbonate tube (C) and running wheel (D). Cage contents were arranged as described in Methods, and as pictured (E–H). (E) Standard caging (484 cm^2 surface area) lacking enrichment items was used to house Cntl animals. (F) Larger caging (980 cm^2) was used for Calm animals and these cages contained a corner-fitting cardboard nest box, shredded nesting material and a polycarbonate tube. (G) Cntl Ex caging (484 cm^2) was similar to Cntl, but included a running wheel. (H) Calm Ex caging was also larger (980 cm^2) and contained a nest box, shredded nesting material, a polycarbonate tube and a running wheel. (I) Experimental timeline; 8-wk-old male BALB/c mice (n = 10 per group) acclimatized to the husbandry facility in standard caging for 14 d. Animals were randomized and placed in respective experimental cages (five per cage) for 70 d. EPM studies were carried out at d 42.

All supplementary materials are available online at www.molmed.org.

RESULTS

Generating a Stress-Reducing Environment

Items that were placed in enhanced cages included a cardboard nest box, shredded paper nesting material, and a polycarbonate mouse tube (Figures 1A–C). Additionally, certain cages contained a silent running wheel (Figure 1D). Eight-week-old BALB/c mice were used in these studies. Although females are known to be less territorial and aggressive, we used male mice to circumvent estrous cycle variability and its effects on hormonal regulation. Mice were randomly assigned to one of four caging groups (n = 10 per group): Cntl, Calm, Cntl Ex or Calm Ex. Cntl animals were group housed (five animals per cage) in standard caging without enhancements (Figure 1E). Calm animals were housed in larger caging containing a cardboard nest box for shelter, paper nesting material to promote nesting behavior, and a polycarbonate tube to mimic tunneling (Figure 1F). Animals in Cntl Ex were housed in standard caging equipped with a running wheel (Figure 1G). Lastly, Calm Ex caging included the same enrichments as Calm with the addition of a running wheel (Figure 1H). See Methods for further details.

Before being placed in their respective experimental caging environments, all mice were kept in Cntl caging for a period of 14 d to allow them to acclimatize to the animal husbandry facility (Figure 1I). After acclimatization, animals were housed in experimental caging, according to their randomized group assignments, until the end of the experiment. On d 42 all animals were subjected to assessment of anxiety-related behavior on an EPM. At the end of the experiment, on d 70, animals were killed for blood collection, *in vitro* immunologic studies, and assessment of body composition. At regular intervals fecal samples were collected from each animal and analyzed for corticosterone metabolites.

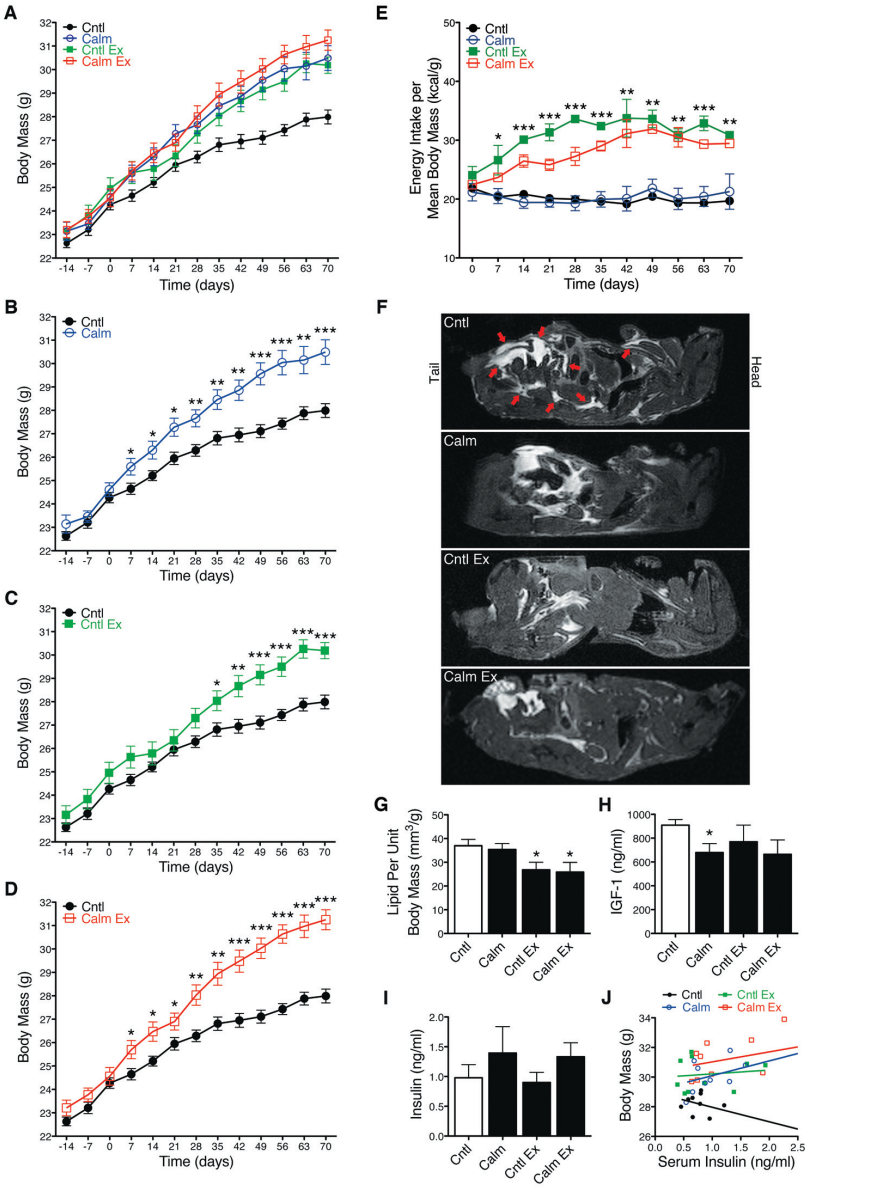

Figure 2. Changes in body mass and metabolism. (A) Calming and exercise resulted in increased mean body mass (time*Calm, $P < 0.0001$; time*exercise, $P < 0.0001$). (B) Body mass of Calm mice was significantly ($P < 0.05$) greater after 7 d, compared with Cntl, and this difference grew over time. (C) Cntl Ex animals exhibited greater body mass than Cntl after 35 d (35 d, $P < 0.05$). (D) Calm Ex animals showed greater body mass after 7 d ($P < 0.05$) and this difference also increased. (E) Cntl and Calm animals had similar weekly energy intake, whereas Cntl Ex and Calm Ex animals displayed significantly greater energy intake, compared with Cntl. (F) MRI visualization of mouse lipid (red arrows). Cntl and Calm animals had similar amounts of lipid, whereas Cntl Ex and Calm Ex animals had less visible lipid. (G) Quantification confirmed that Cntl and Calm animals had similar adiposity, whereas Cntl Ex and Calm Ex animals were significantly leaner than Cntl (Cntl Ex, Calm Ex, $P < 0.05$). (H) Serum IGF-1 levels in Calm mice were lower than in Cntl ($P < 0.05$). (I) Serum insulin levels were higher in Calm and Calm Ex animals compared with Cntl (not significant). (J) Calm and Calm Ex animals displayed positive correlations between insulin and body mass and their slope differed significantly from Cntl (Calm, $P < 0.001$; Calm Ex, $P < 0.05$). Data represented as mean ± standard error of the mean (SEM). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

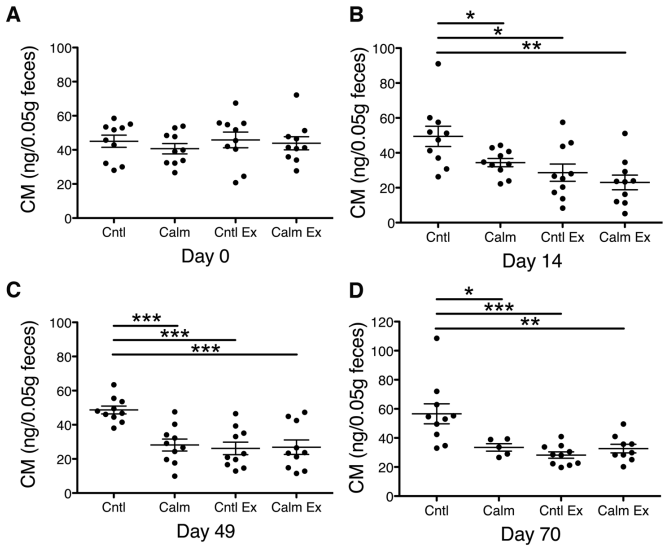

Figure 3. Calm and exercise caging reduce stress hormone production. To assess biological stress levels we used an established noninvasive technique in which we measured CM levels in mouse fecal pellets (see Methods). (A) At d 0, before animals were placed in experimental caging, mean CM levels were similar in all groups. (B) After 14 d of housing in experimental caging, Calm, Cntl Ex and Calm Ex animals had significantly lower fecal CM levels compared with Cntl animals (Calm, Cntl Ex, $P < 0.05$; Calm Ex, $P < 0.01$). (C) At d 49, fecal CM levels from experimental animals had decreased further (Calm, Cntl Ex, Calm Ex, $P < 0.0001$). (D) At the 70-d endpoint of the experiment, fecal CM levels in experimental animals remained significantly lower than Cntl (Calm, $P < 0.05$; Cntl Ex, $P < 0.001$; Calm Ex, $P < 0.01$); note the change in scale to accommodate outlier. Data represented as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Calming and Exercise Increased Body Mass

Body mass was monitored weekly in all groups. We found that both calming and exercise resulted in significantly (time*Calm, $P < 0.0001$; time*exercise, $P < 0.0001$) increased body mass (Figure 2A). After 7 d of experimental caging, Calm animals exhibited a significantly greater mean body mass compared with Cntl (7d, difference in means [M_d] 0.94 g, 95% confidence interval [CI] 0.04–1.84, $P = 0.041$), and this difference increased (70 d, M_d 2.50 g, CI 1.23–3.77, $P = 0.0006$) over the course of the experiment (Figure 2B). Animals housed in Cntl Ex caging also had a greater mean body mass than Cntl animals (35d, M_d 1.23 g, CI 0.16–2.37, $P = 0.027$) (Figure 2C). Similar to Calm, Calm Ex animals showed a greater mean body mass than Cntl animals (7 d, M_d 1.05 g, CI 0.10–2.00, $P = 0.032$), and increased the difference over the duration of the experiment (70 d, M_d 3.26 g, CI 2.17–4.35, $P < 0.0001$). Collectively, these findings suggest that both Calm caging and voluntary exercise can induce rapid gains in body mass in growing mice.

Energy Intake Was Affected by Exercise

To further characterize the differences in body mass described above, we investigated changes in energy intake, body composition and peptide hormone levels in experimental and control animals (Figures 2E–J). Dry food consumption was monitored and recorded weekly by cage for the length of the experiment. These data are expressed as weekly cage-total energy intake per mean cage body mass (kcal/g) at the end of each week to account for longitudinal increases in body mass. Remarkably, although we observed similar proportional weekly energy intake between the Calm and Cntl groups (Figure 2E), Calm animals exhibited significantly greater body mass than Cntl animals (Figure 2B). We also observed that Cntl Ex and Calm Ex animals consumed greater quantities of food per week than Cntl and Calm animals (Figure 2E). In comparing the two exercise groups, we noted that for time points 14, 21, 28 and 35 d, Cntl Ex animals displayed greater energy intake (Figure 2E) but less body mass (Figure 2A) than Calm Ex animals, although these differences did not reach statistical significance.

Calm Caging Had Little Effect on Body Composition, whereas Exercise Reduced Adiposity

To determine whether the increased body mass in experimental groups was attributable to differences in body composition, we carried out an MRI protocol (Figures 2F, G). Animals were imaged by using a three-dimensional fast-spin–echo sequence that emphasized the lipid signal contrast (Figure 2F, red arrows), facilitating subsequent adipose tissue segmentation and quantification (see Methods).

Cntl and Calm animals had similar quantities of lipid, whereas Cntl Ex and Calm Ex animals had less visible lipid than Cntl animals (Figure 2F). Quantification of the proportion of adipose tissue was expressed as total lipid volume per total body mass. These data revealed that Cntl and Calm animals had comparable adiposity (M_d 1.56 mm^3/g, CI –6.16 to 9.29) and similar body composition (Figure 2G). Cntl Ex and Calm Ex animals, however, exhibited a significantly smaller proportion of adipose tissue compared with Cntl (Cntl Ex, M_d –10.15 mm^3/g, CI –18.87 to –1.41, $P = 0.025$; Calm Ex, M_d –11.11 mm^3/g, CI –21.37 to –0.85, $P = 0.035$), but were similar to each other (Figure 2G). Together, these findings suggest that increased body mass in Cntl Ex and Calm Ex animals may be partially attributable to increased lean muscle mass as a result of voluntary wheel running. However, Cntl and Calm animals appeared to have sim-

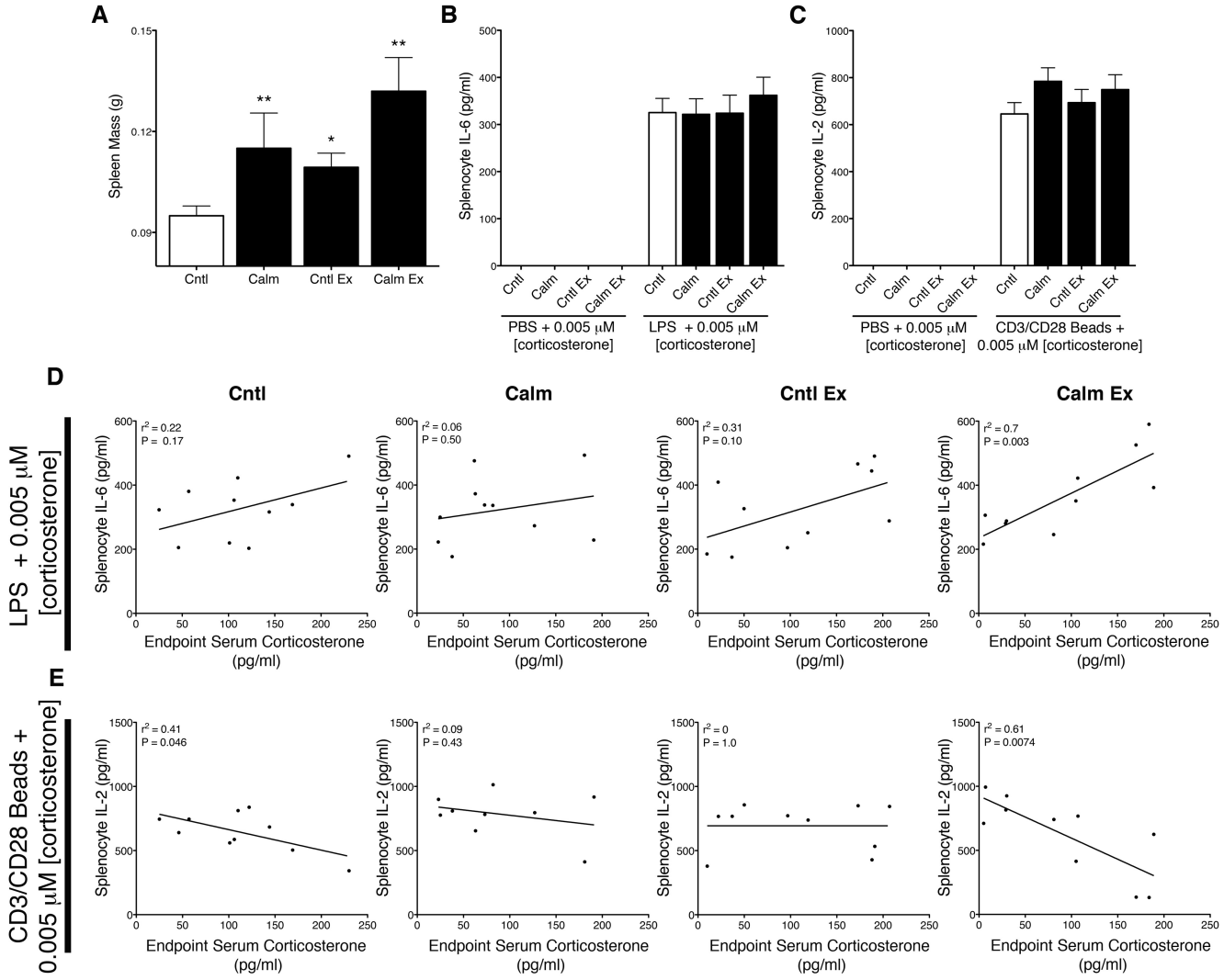

Figure 4. Caging environment affects the murine immune system. At the endpoint of the experiment animals were sacrificed and spleens were taken for massing (A). Splenocytes were isolated as described in Methods, and were resuspended in medium containing 5% charcoal-stripped FBS, which has minimal hormone content. Cultures were treated with LPS (1 μg/mL), anti-CD3 plus anti-CD28 (CD3/CD28) beads (1 bead/cell), or phosphate-buffered saline for 18 h. Some wells contained varying concentrations of corticosterone to assess glucocorticoid-mediated suppression of cytokine production. Data displayed are from cultures containing 0.005 μmol/L corticosterone, because this concentration is nearest to physiological levels (B–E). (A) Spleen mass for Calm, Cntl Ex and Calm Ex mice was greater than that for Cntl mice (Cntl Ex, $P < 0.05$; Calm, Calm Ex, $P < 0.01$). (B) In all groups, LPS-treated splenocytes produced comparable levels of IL-6. (C) anti-CD3/anti-CD28 stimulation of splenocytes from Calm animals exhibited slightly greater production of IL-2 than Cntl (not significant), whereas Cntl Ex and Calm Ex animals showed little difference from Cntl. (D) LPS-stimulated splenocyte production of IL-6 was positively correlated with serum corticosterone level at the time that animals were killed, but this relationship was significant only for Calm Ex animals ($r^2 = 0.7$; $P < 0.01$). (E) Conversely, CD3/CD28 bead stimulation of IL-2 production was negatively correlated with serum corticosterone level at the time that animals were killed. This relationship was significant in Cntl and Calm Ex animals (Cntl, $r^2 = 0.41$, $P < 0.05$; Calm Ex, $r^2 = 0.61$, $P < 0.01$). Data represented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

ilar body composition, a finding that provided little explanation of how Calm mice gained significantly more body mass while maintaining the same proportional energy intake as Cntl animals.

Effects of Calming on Peptide Hormones

To determine whether Calm-associated body mass differences were related to circulating hormone concentrations, we collected serum samples at the 70-d endpoint and assayed them for IGF-1 and insulin (Figures 2H, I). IGF-1, a focal mediator of growth hormone, is produced by the liver and can stimulate growth in

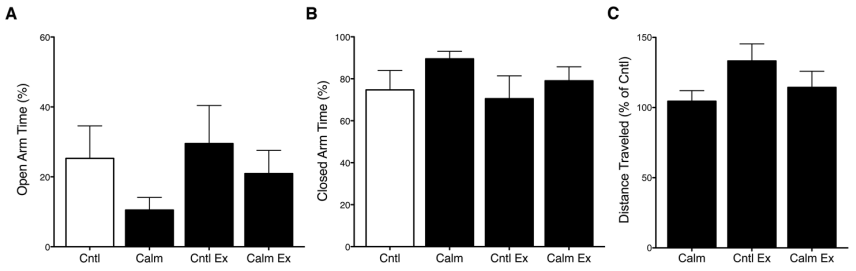

Figure 5. Calm caging and behavior in the EPM test. At d 42 we used an EPM (see Methods) to assess behavioral changes in mice housed in each of the four caging conditions (A–C). (A) Calm animals spent less percent time in open arms of the EPM than Cntl and Cntl Ex animals (not significant). (B) Percent time spent in the closed arms was similar in all groups. (C) Cntl, Calm and Calm Ex mice traveled similar mean distances during EPM observation, whereas Cntl Ex animals traveled a greater mean distance (not significant) compared with the other groups (data expressed as distance traveled per animal/mean distance traveled by Cntl animals). Data represented as mean ± SEM.

most cell types, including muscle, bone and connective tissue. Previous studies of enriched environments have reported upregulation of IGF-1 in the rodent central nervous system (39), and thus, we hypothesized that serum IGF-1 might be upregulated as a product of Calm caging or exercise. We found, however, that compared with Cntl animals, serum levels of IGF-1 were lower in Cntl Ex (M_d –278 ng/mL, CI –897 to 342) and Calm Ex (M_d –489 ng/mL, CI –1032 to 54) and significantly lower (M_d –459 ng/mL, CI –832 to 86, $P = 0.019$) in Calm animals (Figure 2H). We also measured serum levels of insulin, the principal hormone responsible for regulating glucose metabolism. We found that both Calm and Calm Ex animals exhibited trends toward higher levels of serum insulin compared with Cntl animals (Calm, M_d 0.42 ng/mL, CI –0.06 to 1.46; Calm Ex, M_d 0.28, CI –0.10 to 0.65), although the results lacked statistical significance (Figure 2I). Given the functional role that insulin plays in metabolism and energy homeostasis, we reasoned that higher levels of insulin might be associated with enhanced ability to derive energy from digested dry food. To examine this relationship, we looked at the association between serum insulin level and final body mass within each group of our study. We found that a linear regression for Cntl animals revealed a negative relationship between serum insulin level at the end of the experiment and final body mass (Cntl insulin effect –0.98 g per ng/mL of insulin, CI –0.40 to –1.56, $P = 0.0018$) (Figure 2J). However, Calm, Cntl Ex and Calm Ex regressions had positive effects, indicating that higher insulin levels were associated with greater body mass (Calm insulin effect 0.99 g per ng/mL of insulin, CI 0.58 to 1.41, $P < 0.0001$; Cntl Ex insulin effect 0.26 g per ng/mL of insulin, CI –1.04 to 1.56, $P = 0.69$; Calm Ex insulin effect 0.67 g per ng/mL of insulin, CI –0.43 to 1.78, $P = 0.22$) (Figure 2J). The overall insulin by caging group interaction was statistically significant ($P < 0.0001$). These results suggest that caging-induced changes in body mass were related to altered responses to serum insulin level, although further work is needed to confirm this association.

Both Calming and Exercise Reduced Corticosterone Production

The most widely used biomarker of rodent stress is the production of corticosterone. Collecting blood from an animal to measure circulating corticosterone levels, however, may elicit a substantial stress response. For the purposes of assessing corticosterone production in the present study, we employed a less stressful, noninvasive technique in which we measured CMs found in fecal pellets from each animal (Figure 3). CM measures are well correlated with a graded average of corticosterone production from the preceding 10 h, and these results have been extensively validated in laboratory mice and other species (35–38,40). Fecal pellets were collected from individual mice and were later assayed for CM as described in Methods. We found that on d 0, before animals were placed in their respective experimental caging environments, the mean levels of CMs were similar in all groups (Figure 3A). After mice were housed in Calm, Cntl Ex or Calm Ex caging for 14 d, they exhibited significantly lower fecal CM levels (Calm, M_d 15.1 ng, CI 1.9–28.2, $P = 0.027$; Cntl Ex, M_d 20.8 ng, CI 4.8–36.8, $P = 0.014$; Calm Ex, M_d 26.4, CI 11.4–41.4, $P = 0.0017$) compared with Cntl animals (Figure 3B). On d 49, mean fecal CM levels from experimental animals were even lower (Calm, M_d 20.5 ng, CI 11.7–29.4, $P < 0.0001$; Cntl Ex, M_d 22.5 ng, CI 13.4–31.5, $P < 0.0001$; Calm Ex, M_d 21.8, CI 11.7–32.0, $P = 0.0003$) and this effect persisted through d 70 (Calm, M_d 23.1 ng, CI 1.3–44.9, $P = 0.039$; Cntl Ex, M_d 28.4 ng, CI 13.2–43.5, $P = 0.0010$; Calm Ex, M_d 23.9 ng, CI 7.4–40.4, $P = 0.0070$) the last day of the experiment (Figures 3C, D). These findings suggest that both Calm caging and caging equipped with a running wheel can effectively reduce stress levels as evidenced by lasting reductions in CM production.

Immunologic Changes in Mice Housed in Calm and Exercise Caging

To characterize the effects of Calm and exercise caging on murine immune function, we examined spleens and carried out functional *in vitro* studies using splenocytes (Figure 4). At the experimental endpoint animals were sacrificed and spleens were aseptically removed and massed. We found that both calming and exercise generated a significant increase (Calm, $P = 0.0086$; exercise, $P = 0.049$) in spleen mass (Figure 4A).

Chronic social stress has been shown to affect innate and adaptive immunity

in humans and animals. For example, studies of chronic stress have revealed increased production of IL-6 after lipopolysaccharide (LPS) stimulation of splenocytes (41), and both reduced lymphocyte proliferation and suppressed IL-2 production (5,42). In this work, to assess whether there were changes in innate or adaptive immune function we stimulated splenocyte cultures with LPS (1 μg/mL) or anti-CD3 plus anti-CD28 (CD3/CD28) Dynabeads (1 bead/cell), respectively. Some stimulated splenocyte cultures were treated with different concentrations of corticosterone, to assess leukocyte sensitivity to corticosterone-mediated suppression of cytokine production. All cells were cultured in splenocyte medium containing 5% charcoal-stripped FBS, which minimizes background hormone levels commonly found in serum and reduces unspecific binding of the glucocorticoid receptor. Because murine immune cells usually function in the presence of a small concentration of corticosterone (43), the results displayed are from cultures containing 0.005 μmol/L corticosterone, the most physiologically relevant condition tested (Figures 4B–E).

LPS-stimulated splenocyte cultures from Calm and Cntl Ex animals exhibited comparable levels of IL-6 to those of Cntl animals (Figure 4B). Cultures from Calm Ex animals displayed a slight trend toward greater production of IL-6 after stimulation with LPS, but these data were not statistically significant (Figure 4B). Although splenocyte production of IL-2 in response to CD3/CD28 bead stimulation was similar among Cntl, Cntl Ex and Calm Ex groups, cultures from Calm animals had slightly higher (M_d 138.0 pg/mL, CI –18.8 to 296.1, $P = 0.081$) mean levels of IL-2 (Figure 4C).

Acute Stress and Immunomodulation

Stress is known to be immunomodulatory and may enhance or suppress immune responses depending on the type of stressor, acute or chronic, and the cell populations being studied, innate or adaptive (9,11,44,45). For example, human studies have shown that acute psychological stress results in increased production of IL-6 in LPS-stimulated peripheral blood mononuclear cells (46). In the context of animal studies, the handling of mice before sacrifice is a substantial acute stressor, resulting in increased serum levels of stress mediators such as corticosterone and epinephrine (47). To assess the level of acute stress at the end of the experiment we measured the quantity of corticosterone found in serum samples taken from mice at the time of sacrifice. We observed a distinct pattern, in that within individual cages, the animals killed first often had lower levels of acute stress than subsequent animals, as indicated by serum corticosterone concentration (Supplementary Figure 1). Thus, in each group there was a broad range of serum corticosterone levels at the time of sacrifice (Supplementary Figures 1A–D). The within-group variation in serum corticosterone provided an opportunity to investigate the effects of varying levels of acute stress on functional immunity in animals housed in each of the four caging conditions.

To determine whether the level of acute stress at the time of sacrifice had any effect on cytokine production, we carried out linear regression analyses to examine the relationship between serum corticosterone at the time of death and *in vitro* stimulation of splenocyte cytokine production (Figures 4D, E). We found that all four groups displayed a positive association between serum corticosterone and LPS-stimulated production of splenocyte IL-6, but the correlation was a strong fit and statistically significant ($P = 0.0029$) only for Calm Ex animals (Figure 4D). In contrast, the overall trend for serum corticosterone and IL-2 production after CD3/CD28 bead stimulation was a negative correlation (Figure 4E). Similar to the results of IL-6 analysis, we found that the strongest and most significant ($P = 0.0074$) relationship between serum corticosterone and IL-2 production was within the Calm Ex group (Figure 4E).

To investigate whether the above observations were specific to the presence of 0.005 μmol/L corticosterone in the splenocyte cultures, we applied similar regression analyses to cultures that contained other concentrations of exogenous corticosterone, including: 0, 0.025, 0.05, and 0.075 μmol/L (Supplementary Table 1). These analyses showed that the positive association between serum corticosterone and splenocyte IL-6 production remained intact at several other concentrations of *in vitro* corticosterone and that the strongest relationships were found in Calm Ex animals (Supplementary Table 1). Similarly, the negative correlations observed between serum corticosterone and IL-2 production were found at other concentrations of *in vitro* corticosterone, and again, the Calm Ex group displayed highly significant correlations (Supplementary Table 1).

In summary, animals housed in Calm, Cntl Ex and Calm Ex caging had greater mean spleen mass than Cntl, and our regression analyses suggest that the acute stress of handling had an enhancing effect on innate immune function and a suppressive effect on adaptive immune function as determined by changes in cytokine production. Finally, Calm Ex animals exhibited the greatest sensitivity to acute stress-mediated alteration of immune function.

Caging Environment and EPM Behavior

To assess the behavioral effects of housing animals in enhanced caging, we applied the EPM, a widely used test of rodent anxiety (Figure 5). The EPM is a raised, plus-shaped apparatus consisting of two open arms and two arms with a walled enclosure, or closed arms. The principle of the assay relies on the innate aversion that rodents have to open elevated alleys. Interpretation of EPM behavior is derived from original work showing that rodents given anxiolytic agents spent a greater proportion of time in open arms of the maze (48). In pilot studies of the Calm model, we found that biomarkers of stress decreased to the lowest levels after 6 wks, and we hypothesized that behavioral anxiety might be reduced on a simi-

lar temporal scale. In the present work, at 42 d each animal was placed in the EPM and behavior was digitally recorded for 5 min and later analyzed by a blinded observer (see Methods).

We found that Calm animals spent a smaller proportion of time in open arms of the EPM than Cntl animals, although none of the between-groups comparisons reached statistical significance (Figure 5A). Similarly, the proportion of time spent in the closed arms of the EPM was not statistically significantly different between groups nor were the number of open- or closed-arm entries (Figure 5B, data not shown). Lastly, we measured the total distance traveled by each animal, an indicator of exploration, and found that Cntl Ex animals traveled the greatest distance during the course of the observation period, whereas Cntl, Calm, and Calm Ex mice traversed the maze comparably (Figure 5C).

DISCUSSION

In this paper, we describe an animal model that shows promise as a tool for modeling biological effects of stress reduction. Our data demonstrate that animals housed in stress reduction caging exhibited significant and lasting reductions in fecal CM levels, a measure of physiologic stress (Figure 3). Also, growing animals housed in Calm, Cntl Ex, and Calm Ex caging gained significantly greater body mass than Cntl animals (Figure 2). Strikingly, Calm animals gained greater body mass than Cntl despite comparable weekly energy intake (Figure 2). Lastly, immunologic studies showed that stress-reduction caging was associated with increased spleen mass and that splenocytes from Calm Ex animals were more sensitive to the immunomodulating effects of acute stress (Figure 4).

Animal studies of stress commonly cite corticosterone as a key indicator of stress level and have shown that subjecting mice to chronic mild stress or chronic stress often results in elevations of corticosterone level (49,50). In the present work, we used an established noninvasive method in which we measured fecal corticosterone metabolites from each mouse. This approach allowed us to monitor stress hormones longitudinally without inducing a stress response and also provided us with reliable data that reflect a 10-h graded average of corticosterone production. Our results demonstrated that Calm, Cntl Ex and Calm Ex animals exhibited significant reductions in fecal CM levels compared with Cntl, and that these differences were observed throughout the experiment. Interestingly, calming and exercise were both effective at reducing CM levels. Toward the beginning of the study, at d 14, it appeared that the combination of Calm and exercise resulted in more rapid CM reductions than either approach alone. At subsequent times, however, CM seemed similar in each of the modified caging groups. These findings suggest that a plateau effect on reducing CM may have been reached over time with each of these caging interventions. Collectively, these results show that animals housed in Calm and exercise caging exhibited reduced biomarkers of stress, although, importantly, these results also underscore the relatively higher levels of HPA-axis activity found in Cntl animals, which were housed in standard caging.

Manipulation of the mouse-caging environment can evoke a variety of other measureable physiologic effects. In rodent studies, body mass is easily monitored and widely interpreted as an indicator of health. Chronically stressed animals exhibit substantial reductions in body mass, and disease models often use the extent of body mass loss as a measure of morbidity (51,52). In the present work, we observed greater increases in body mass in animals housed in Calm, Cntl Ex, and Calm Ex caging. We also measured weekly energy intake and, as expected, found that Cntl Ex and Calm Ex mice had greater intake per week compared with Cntl mice. However, after observing the body mass differences between Cntl and Calm animals, we were surprised to find that these groups had very similar weekly energy intake per body mass. Our MRI analyses demonstrated that Cntl and Calm animals had similar proportions of lipid to body mass, indicating similar body composition. We then hypothesized that the body mass changes in Calm mice might be attributable to endocrine changes. Measurement of serum levels of insulin revealed that Calm and Calm Ex animals had higher levels of insulin than Cntl, although the results did not reach statistical significance. Further analysis showed that Calm, Cntl Ex and Calm Ex animals had positive correlations between serum insulin level and final body mass, whereas Cntl animals showed a negative correlation. These results provide evidence for interaction between caging condition and the effects of insulin on body mass. This interaction could be driven by factors such as altered autonomic nervous system or HPA activity, and in subsequent studies we aim to further examine this issue.

Glucocorticoids are known to have catabolic effects on muscle, skin, adipose and connective tissues, which results in decreased synthesis and increased degradation of protein and fat, and reduced glucose and amino acid uptake (53). Taken together, the reduced levels of glucocorticoids combined with increased levels of circulating insulin found in Calm animals may explain how they gained significantly more body mass than Cntl animals without increased energy intake per unit body mass. To validate this hypothesis, further metabolic and endocrine studies are needed.

Our immunologic studies revealed that both calming and exercise resulted in greater mean spleen mass. Calm Ex animals had the greatest spleen mass, which suggests there may be an additive effect of calming and exercise on immune function. Other important work has shown that chronic restraint stress in mice generated marked reductions in spleen cellularity and that this was due to CD95-mediated lymphocyte apoptosis (10). Together, these findings may suggest that animals in reduced-stress caging exhibit greater spleen mass as a product of reduced leukocyte apoptosis.

In our *in vitro* work, we found that the acute stress of handling before sacrifice increased the production of splenocyte IL-6 after stimulation with LPS and the decreased production of IL-2 after CD3/CD28 bead stimulation. These results confirm previous reports in humans that acute stress can exert enhancing effects on innate immunity (46). Although these trends were apparent across all groups, we found that the relationships between acute stress and modulation of cytokine production were the strongest for Calm Ex animals. These data support the concept that Calm Ex animals had increased sensitivity to acute stress-associated immune modulation and that this sensitivity may be a result of reduced chronic exposure to corticosterone, catecholamines and other stress-related factors during the course of the experiment.

Analysis of the EPM data suggested little difference between groups in anxiety-related behavior. Interestingly, other investigators have found that after exposures to similar enriched caging environments, BALB/c mice spent less time in the open arms of the EPM (54), whereas C57BL/6 mice spent greater time in the open arms (25), suggesting the possibility that mouse strain may interact with the environment to influence maze behavior. Additionally, based on the characterization of BALB/c as a relatively anxious mouse strain (55), detection of changes in anxious behavior with adequate sensitivity may require the application of alternative tests of rodent anxiety. In planned future studies, we will assess anxiety-related behavior in BALB/c mice using a light/dark test and open-field test and, separately, we plan to house other strains of mice, such as C57BL/6, in a Calm environment to further characterize the importance of mouse strain on Calm-related reduction of anxious behavior.

The use of murine models for studying biological systems and disease is ubiquitous in scientific research. Researchers commonly conduct mouse studies with the goal of providing insights that can be applied to improve human health. However, results in animal models often do not translate successfully to clinical applications. There are a variety of reasons why the translation fails, with species differences being a central explanation. Our results and reports from other groups (56,57) raise the possibility, however, that standard caging conditions used in most experimental settings are a source of chronic mild stress that may contribute to problems in translating murine research into human studies. Within this framework, cage density, lack of species-specific behaviors, and continuous visibility would be sources of persistent stress. We have shown that caging environments that mimic elements of a natural murine habitat and provide access to an exercise wheel can substantially influence various aspects of mouse physiology. The caging conditions we have studied may represent a "healthier" mouse environment. To enhance the degree to which animal studies successfully translate to clinical applications, it may be worth investigating whether standard housing conditions are influencing the accuracy of results rooted in mouse research, particularly when outcomes that may be influenced by stress are involved.

CONCLUSION

In summary, we describe an animal model that can be used to study the biological effects of stress reduction. This technique will allow us, and others, to characterize the pathways related to stress reduction that are important for influencing health outcomes. This approach can also be applied in conjunction with other model systems of disease, such as neurodegeneration or viral infection models, to determine the relevance of stress reduction on clinical course and pathology. Lastly, we predict that pivotal insights, concerning stress and human health, will result from comparing Calm mouse findings with results from clinical studies of stress reduction.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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Supplemental Data

The Calm Mouse: An Animal Model of Stress Reduction

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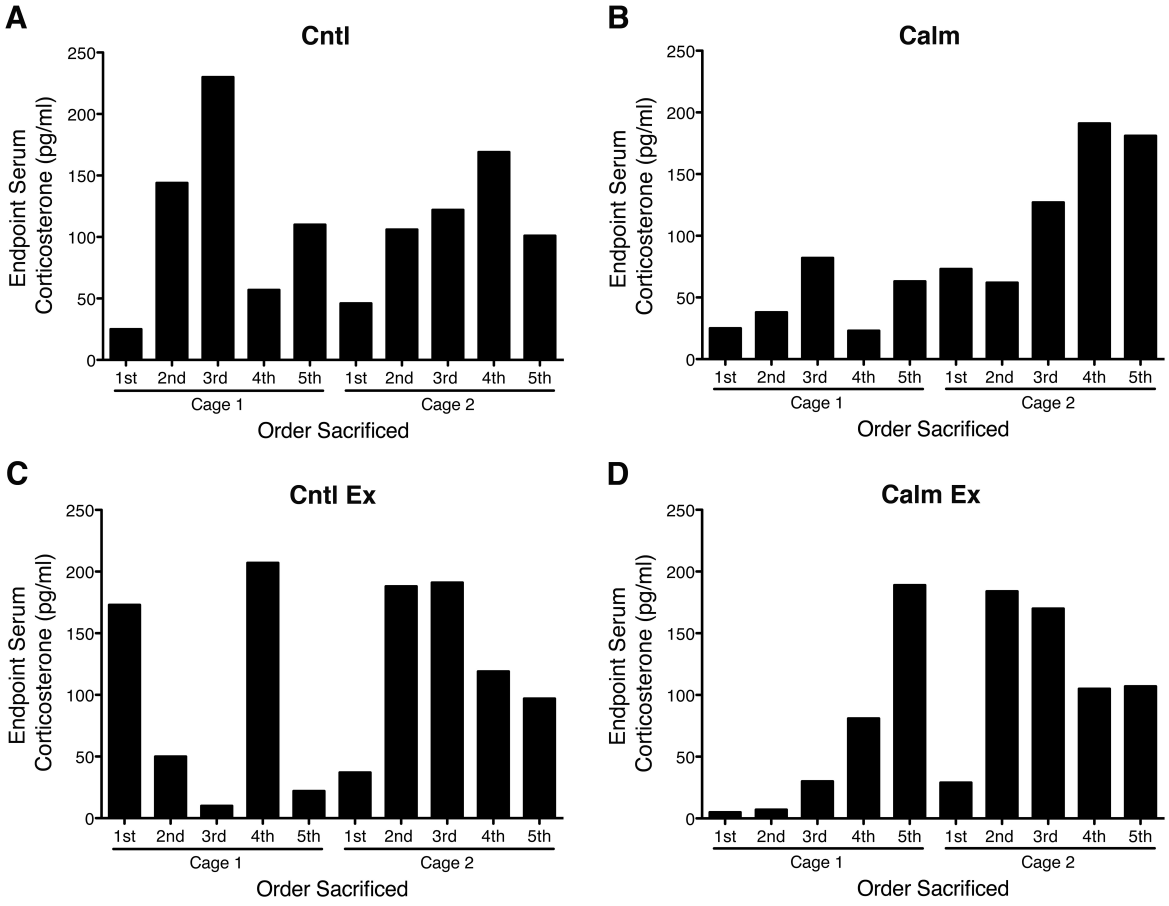

Supplementary Figure 1. Acute stress of handling before sacrifice induces increased serum corticosterone levels. At the endpoint of the experiment, the order of animal sacrifice was recorded and serum samples were taken for later measurement of corticosterone concentration. In several instances, we found that, within a cage, animals sacrificed first had lower levels of serum corticosterone than subsequent animals (A-D). 10-15 min elapsed between sample collection for first and fifth animals per cage. Serum corticosterone levels of animals from Cntl (A), Calm (B), Cntl Ex (C), Calm Ex (D), in the order they were sacrificed, demonstrate the similarly broad range of handling-associated acute stress responses between groups.

Supplementary Table 1. Correlation between serum corticosterone at sacrifice and cytokine produciton after splenocyte stimulation.

LPS Stimulation: IL-6 Production & Endpoint Serum Corticosterone					CD3/CD28 Bead Stimulation: IL-2 Production & Endpoint Serum Corticosterone				
Group	*in vitro* Corticosterone (μM)	Slope	r^2	P	Group	*in vitro* Corticosterone (μM)	Slope	r^2	P
Cntl	0	0.5834	0.1180	0.3311	Cntl	0	-1.3322	0.2982	0.1025
Cntl	0.005	0.7378	0.2236	0.1675	Cntl	0.005	-1.6004	0.4105	**0.0460**
Cntl	0.025	0.7097	0.1751	0.2288	Cntl	0.025	-1.0920	0.4312	**0.0391**
Cntl	0.05	0.4986	0.1021	0.3681	Cntl	0.05	-0.7111	0.2305	0.1602
Cntl	0.075	0.5987	0.1237	0.3189	Cntl	0.075	-0.3063	0.0418	0.5711
Calm	0	0.5814	0.0520	0.5264	Calm	0	-1.6528	0.2404	0.1803
Calm	0.005	0.4225	0.0596	0.4967	Calm	0.005	-0.8251	0.0918	0.4280
Calm	0.025	0.4009	0.0352	0.6037	Calm	0.025	-1.5652	0.2747	0.1475
Calm	0.05	0.2300	0.0255	0.6596	Calm	0.05	-0.5986	0.0683	0.4971
Calm	0.075	0.2062	0.0272	0.6491	Calm	0.075	-1.3617	0.3795	0.0773
Cntl Ex	0	0.9980	0.3373	0.0783	Cntl Ex	0	-0.1133	0.0028	0.8845
Cntl Ex	0.005	0.8760	0.3081	0.0958	Cntl Ex	0.005	-0.0002	0.0000	0.9998
Cntl Ex	0.025	0.7819	0.2870	0.1105	Cntl Ex	0.025	-0.3822	0.0177	0.7139
Cntl Ex	0.05	0.7387	0.2619	0.1305	Cntl Ex	0.05	0.0208	0.0001	0.9807
Cntl Ex	0.075	0.7682	0.3316	0.0815	Cntl Ex	0.075	-0.1193	0.0038	0.8663
Calm Ex	0	1.4731	0.6385	**0.0056**	Calm Ex	0	-2.4349	0.3015	0.1002
Calm Ex	0.005	1.4000	0.6915	**0.0029**	Calm Ex	0.005	-3.2912	0.6133	**0.0074**
Calm Ex	0.025	1.2696	0.8000	**0.0005**	Calm Ex	0.025	-2.5403	0.6085	**0.0078**
Calm Ex	0.05	0.8077	0.7006	**0.0025**	Calm Ex	0.05	-2.9606	0.6906	**0.0029**
Calm Ex	0.075	0.7417	0.6897	**0.0029**	Calm Ex	0.075	-1.5275	0.3699	0.0621

Significant P values shown in bold.