Research Article

Housing in the Animal Enclosure Module Spaceflight Hardware Increases Trabecular Bone Mass in Ground-Control Mice

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ABSTRACT

During spaceflight, mice are housed in specially designed cages called the Animal Enclosure Module (AEM). Utilization of this flight hardware may affect the skeletal properties of housed animals, independent of microgravity considerations. To address this issue, we studied the effect of 13 days of AEM housing versus standard vivarium enclosure on female C57BL/6J mice (n=12/group). The effects of AEM housing were most pronounced in the trabecular compartment. AEM mice had 44% and 144% greater trabecular bone volume fraction and connectivity density, respectively, versus vivarium. A similar response was seen at the proximal humerus. We noted a decrease in proximal tibia osteoclast surface (-65%) and eroded surface (-73%) for AEM versus vivarium, while tibia trabecular mineralizing surface (MS/BS) was nearly three-fold greater. Surprisingly, there was also decreased osteoblast surface, as well as lower osteoid volume, surface, and thickness at this site. The effects of AEM housing on femur cortical bone were modest: there was greater periosteal MS/BS, with no effect at the endocortical surface, and lower femur stiffness. Taken together, we have demonstrated significant effects of AEM housing on ground control mice, particularly in the trabecular bone compartment. These findings suggest that an early increase in bone formation, perhaps due to altered behavior and loading in this unique housing environment, was followed by decreased bone formation and resorption as the animals adapted to their new environment. Characterization of spaceflight animal housing is critical to elucidating the true effects of microgravity on skeletal parameters and for the proper selection of ground-based controls.

INTRODUCTION

The “weightless” environment encountered during spaceflight complicates the traditional concerns involved in animal housing, including nutrient delivery and waste management. Spaceflight experiments utilizing mice or rats...
require habitats that are specifically designed for this environment. During studies on the space shuttle, rodents are housed in a specially designed stainless steel meshed cage called the Animal Enclosure Module (AEM) (Brooks, 1981). Despite the retirement of the shuttle program in 2011, plans are in place to adapt the AEM for use on the International Space Station (ISS). AEMs are equipped with systems that provide food and water *ad libitum* (Zerath et al., 2002). In addition, these flight enclosures have a constant airflow mechanism designed to move free-floating animal waste towards an exhaust filter. AEMs allow for efficient containment of animals in the confines of the crew cabin, while also satisfying their homeostatic demands. The first test flight of the AEM was during shuttle mission STS-8 in 1983 (Smith et al., 1987). Since then, AEMs have been successfully utilized on over 20 missions involving both rats and mice.

Despite their demonstrated utility, AEM housing may have an effect on the physiology of the rodents contained within. Beyond consideration of the spaceflight environment, a non-standard cage environment like that found with AEMs may lead to unexpected changes in animal behavior that could complicate experimental results. These considerations are especially important in studies examining the skeletal effects of microgravity. In this case, even subtle changes in animal loading can distort experimental findings. For example, a study by Morey-Holton and colleagues revealed that group housing of rats in AEMs reduced their skeletal response to spaceflight by as much as 80% (Morey-Holton et al., 2000).

In the present study, we compared the effects of 13 days of AEM housing on various bone compositional, microarchitectural, and histological parameters. We documented significant effects of the AEM on the skeletal properties of mice, particularly in the trabecular bone compartment. This study was conducted as part of a ground-based parallel of an animal payload on space shuttle flight STS-108. Characterization of animal housing is important so that results from spaceflight experiments can be more reliably compared to ground-based controls and studies from other missions.

**MATERIALS AND METHODS**

**Ethics Statement**

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The protocol for this study was approved by the Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA) Kennedy Space Center (Protocol #01-028-1).

**Animals**

This thirteen-day experiment, conducted on the ground, modeled the flight experiment profile of the CBTM-01 payload on STS-108. 64 day-old female C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME, USA) were utilized for this study. Mice were assigned to standard vivarium cages or AEM flight hardware at n=12/group. A group of baseline mice (n=12) was sacrificed on day 0. AEM and vivarium mice received an intraperitoneal injection of the fluorescent bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study.

**Flight Hardware & Housing**

On the space shuttle, mice are maintained in AEM hardware provided by NASA Ames Research Center (Moffett Field, CA, USA) (Figure 1). The AEMs provide rodent food (NASA Rodent Diet TD97071, Harlan-Teklad; Wisconsin, USA) and water *ad libitum* (Zerath et al., 2002). Because urine and feces are free to float around the cage in microgravity, the AEMs have a constant airflow mechanism designed to move waste towards an exhaust filter. The dimensions of the AEM are 24.50 x 43.69 x 51.05 cm and it weighs approximately 27.2 kg with food, water, and animals. The total floor space with the water box installed is 645 cm$^2$. The AEM can support up to five adult rats or ten adult mice. For the present study, housing density was within NIH guidelines with approximately 81 cm$^2$ floor area per mouse (eight mice per AEM, with a divider separating half the mice). AEMs were contained within the Orbiter Environmental Simulator (OES) at NASA’s Life Science Support Facility (“Hangar L”) at Cape Canaveral Air Force Station (Cape Canaveral, FL, USA). The OES creates an...
environment that mimics the temperature, humidity, and CO\textsubscript{2} levels within the cabin of the space shuttle. The parameter of primary concern was atmospheric CO\textsubscript{2}, which averaged more than 3000 ppm during the STS-108 flight. This level is normal in spacecraft and is approximately ten times that of a well-ventilated room on Earth. The constant airflow mechanism described above was activated for this ground-based study, although it was not able to clear waste in normal gravity. Four internal lamps provided an average 14 lux of illumination with timers to provide 12-hour light/dark cycles.

The vivarium-housed animals were maintained in the Animal Care Facility at Hangar L with standard environmental conditions, feeding mechanisms, and a 12-hour light/dark cycle. Exact space shuttle environmental conditions (i.e., temperature, humidity, CO\textsubscript{2}) were not reproduced for vivarium-housed mice. Each cage had a floor area of 522 cm\textsuperscript{2} and animals were group housed (4 mice/cage; 130 cm\textsuperscript{2} of floor area per mouse) with access to the same rodent diet and water \textit{ad libitum} as their AEM-housed counterparts.

**Necropsy**

Baseline animals were sacrificed at the start of the study. After 13 days of housing, vivarium and AEM-housed mice were sacrificed. All animals were weighed and then anaesthetized with isoflurane (2\%). Mice were then sacrificed by exsanguination via cardiac puncture and cervical dislocation. Hindlimbs and forelimbs were removed and the tibia, femur, humerus, and lumbar vertebrae of each animal were isolated and cleaned of all non-osseous tissue. The left femur,
required for mechanical testing and mineral composition analysis, was allowed to air-dry. The right femur, left and right tibia, right humerus, and L5 lumbar vertebrae were fixed in a 10% neutral buffered formalin solution for 48 hours, rinsed with distilled water, and stored in 70% ethanol. These bones were utilized for microcomputed tomography analysis, histology, and histomorphometry as described below.

**Microcomputed Tomography**

Trabecular bone architecture was analyzed using microcomputed tomography (µCT20; Scanco Medical AG; Brüttisellen Switzerland) with an isotropic voxel size of 9 μm with scan settings of 55 KVP, 145 mA, and 200 ms integration time. Trabecular microarchitecture was analyzed with Scanco software immediately distal to the epiphyseal plate in the right proximal tibia and humerus. Trabecular bone evaluation was performed on 100 slices (0.9 mm total) for each tibia and 70 slices (0.6 mm total) for each humerus, producing three-dimensional images for analysis. Bone morphometric parameters were then quantified using Scanco software. In accordance with published guidelines (Bouxsein et al., 2010), trabecular bone parameters included trabecular bone volume fraction (BV/TV), connectivity density (Conn.D), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and structure model index (SMI).

**Cortical Histomorphometry**

After ethanol fixation, the right femurs were air-dried and embedded in non-infiltrating Epo-Kwick epoxy (Buehler; Lake Bluff, IL, USA). These epoxy disks were sectioned in half with a low-speed saw (Buehler; 12.7 cm x 0.5 mm diamond blade) at the mid-diaphysis of the femur. The proximal half was wheel-polished to a flat, smooth surface with 600-, 800-, and 1200-grit carbide paper, followed by a cloth impregnated with 6 μm diamond paste. This allowed micrographs at 50x magnification to be taken of the femur cross-section under a blue light (400 nm). Green Calcein labels were visualized, indicating the bone formation sites present during the study. Quantitative histomorphometric analysis was performed using these photographs and SigmaScan Pro software (SPSS; Chicago, IL, USA).

Measurements of bone morphology (Dempster et al., 2013) included tissue volume (TV) enclosed by the periosteal bone surface (Ps.BS) and volume of the marrow cavity (Ma.V) enclosed by the endocortical bone surface (Ec.BS). Bone volume (BV) was calculated as TV - Ma.V. Cortical thickness (Av.Ct.Th) was measured at the medial, lateral, posterior, and anterior location of the femur diaphysis and averaged.

Due to the absence of double Calcein labels, we were not able to accurately determine mineral apposition rate (MAR) or bone formation rate (BFR) (Foldes et al., 1990). As a result, we used the mineralizing surface (MS/BS) as an index of bone formation as described previously (Grimston et al., 2011). The linear content of the Calcein labeled perimeter was defined as mineralizing surface (MS) and normalized to bone surface (BS) at both Ps and Ec surfaces (i.e., Ps.MS/BS and Ec.MS/BS).

The proportional endocortical eroded surface (Ec.ES/BS) was measured by quantifying the portion of the non-labeled surface with a rough/ruffled border and dividing it by BS.

**Biomechanical Properties**

In order to simulate *in vivo* properties, the air-dried left femurs were rehydrated in phosphate-buffered saline for 90 minutes prior to evaluation (Broz et al., 1993). Three-point bending tests were performed using an Instron 5582 (Instron Corporation; Norwood, MA, USA). Femurs were tested to failure with a 9 mm span length and a deflection rate of 5 mm/min. All bones were tested in the same orientation: the single-point load was applied mid-diaphysis on the anterior surface. The maximal force (F_m; N) and deflection at F_m (δ_m; mm) were measured for all mechanically tested bones. These two properties were also determined at the elastic limit (F_e, δ_e) and the failure point. Stiffness (N/mm) was calculated from elastic force/elastic deflection (F_e/δ_e).

Two-dimensional, cross-sectional moments of inertia of the right femur mid-diaphysis (I_X and I_Y; mm^4) were also calculated. These values were determined using micrographs and the assumption that the periosteal and endocortical surfaces were
in the approximate shape of concentric ellipses (Simske et al., 1992).

Right femurs prepared for histomorphometric analysis were utilized for testing the material properties of the femur diaphysis by microhardness indentation. Three microhardness indents were placed in extant bone within each sectioned and polished femur cross-section using a pyramid-shaped Vicker's diamond indenter (Fischer Scope-H1100 and WINHCU 1.3 software, Fischer Technology; Windsor, CT, USA) with a 50 g load for 10 seconds. In order to minimize edge effects, one indent length was maintained between the indent site, sample edges, and visible lacunae. Pyramid diagonal lengths were measured (250 μm), and the Vicker's hardness number (VHN; kgf/mm²) was calculated using the formula: $VHN = \frac{2F\sin(x/2)}{d^2}$, where $F$=applied load, $x$=pyramid angle (136°), and $d$=average measure of the two diagonal lengths.

Osteoblast and Osteoclast Identification

Following microCT analysis, the right tibias and L5 vertebrae were decalcified using a formic acid solution (Immunocal; Decal Chemical Corporation, Talman, NY, USA) and embedded in a methylmethacrylate resin (ImmunoBed; Polysciences, Warrington, PA, USA) as described previously (Chappard et al., 1987; Erben, 1997). The samples were cut into sagittal sections with a thickness of 3 μm using a microtome (Leica Microsystems, model RM2165; Witzlar, Germany). Each slide was stained with TRAP using a commercial kit (Sigma; St. Louis, MO, USA) to identify osteoclasts and counterstained with hematoxylin to identify osteoblasts. These sections were also used to quantify osteoid volume normalized to total volume (OV/TV), osteoid surface normalized to bone surface (OS/BS), and osteoid thickness (O.Th).

Trabecular Histomorphometry

The left tibias were embedded in a methylmethacrylate resin (Osteo-Bed; Polysciences, Warrington, PA, USA) and cut into sagittal sections with a thickness of 5 μm using a tungsten carbide blade. Slides were left unstained. Quantitative histomorphometric analysis was performed using SigmaScan Pro software on the micrographs captured at 10× magnification under a UV light (400 nm). Histomorphometric evaluation was performed throughout the metaphysis, starting approximately 0.25 mm distal from the growth plate and extending a further 0.5 mm. MS/BS and ES/BS were quantified in the same manner as for cortical bone.

Bone Mineral Composition

Mineral-content analysis was performed on left femurs fractured during mechanical testing. Prior to analysis, the enlarged ends of the femurs were separated where the distal and proximal metaphysis joins the diaphysis. Mineral content data was obtained separately from these bone ends and the diaphysis itself. A properly calibrated analytical scale (Mettler Toledo UMT2; Columbus, OH, USA) was used for all measurements. Dry mass (Dry-M) was measured after heating the bones to 105°C for 24 hours. Mineral mass (Min-M) was measured after the bones had been heated at 800°C for an additional 24 hours. Organic mass (Org-M) was calculated as the difference between the two (Org-M = Dry-M - Min-M). Percent mineralization was calculated as: %Min = (Min-M)/(Dry-M)*100.

Serum Chemistry

At sacrifice, samples of whole blood were collected by cardiac puncture and serum was separated. The concentration of various bone turnover markers was determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits. ELISAs were performed for the bone resorption marker tartrate-resistant acid phosphatase 5b (TRAP5b) (ImmunoDiagnostic Systems Inc; Fountain Hills, AZ, USA), as well as the bone formation markers osteocalcin and alkaline phosphatase (Biomedical Technologies Inc; Stoughton, MA, USA). All ELISA procedures were performed according to the manufacturers’ protocols. Calcium and phosphorus levels were also determined using a Hitachi 717 Automatic Chemistry Analyzer (Roche Diagnostics; Indianapolis, IN, USA).

Statistics

Statistics were completed using Prism 5.0 software (GraphPad Software Incorporated; La Jolla, CA, USA). Statistical comparisons were made via one-way-ANOVA between baseline,
vivarium, and AEM groups with a Student Newman-Keuls post-hoc test. Type I error was set at 95% (p<0.05). All data are reported at mean ± standard error of the mean (SEM). Unless otherwise indicated, all reported changes represent significant differences (p<0.05). Generally, differences between vivarium and AEM groups will be reported and discussed. Differences in relation to baseline control mice are presented to aid in determining if differences between vivarium and AEM are related to normal growth or hardware effects.

RESULTS

Animal Mass

There was no significant difference between any of the groups with respect to total body mass at the start of the study (baseline: 17.5 ± 0.1 g, vivarium: 17.5 ± 0.2 g, AEM: 17.7 ± 0.1 g) or at the end of the study (vivarium: 18.6 ± 0.2 g, AEM: 18.6 ± 0.2 g) (p>0.05 for all).

Mechanical, Material, and Structural Assays

Mechanical stiffness was 18% lower for AEM-housed mice versus vivarium control (p<0.05) (Table 1). There were no other significant differences between AEM and vivarium for any of the parameters obtained from the mechanical, material, and geometric assays that were conducted. Stiffness, elastic force, and maximal force were similarly and significantly greater in vivarium and AEM-housed animals when compared to baseline (p<0.05). Analysis of calculated resistance to torsion revealed significantly greater $I_{\text{max}}$ in AEM-housed mice compared to baseline (p<0.05), with no difference for vivarium. There was no difference in microhardness values between baseline mice and either housing environment.

Table 1. Mechanical, material, and structural properties of the femur. Tissue was collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with $V$ or $A$, respectively (p<0.05).

<table>
<thead>
<tr>
<th>Property</th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stiffness (N/mm)</td>
<td>42.3 ± 2.5$^{VA}$</td>
<td>59.1 ± 2.7$^V$</td>
<td>50.3 ± 2.1$^V$</td>
</tr>
<tr>
<td>Elastic Force (N)</td>
<td>8.6 ± 0.4$^{VA}$</td>
<td>11.2 ± 0.4</td>
<td>11.4 ± 0.3</td>
</tr>
<tr>
<td>Maximal Force (N)</td>
<td>10.3 ± 0.3$^{VA}$</td>
<td>12.6 ± 0.3</td>
<td>12.5 ± 0.3</td>
</tr>
<tr>
<td>Failure Force (N)</td>
<td>8.6 ± 0.4</td>
<td>7.9 ± 0.6</td>
<td>9.0 ± 0.7</td>
</tr>
<tr>
<td>$I_{\text{max}}$ (mm$^4$)</td>
<td>184 ± 9$^A$</td>
<td>213 ± 8</td>
<td>238 ± 6</td>
</tr>
<tr>
<td>$I_{\text{min}}$ (mm$^4$)</td>
<td>97 ± 4</td>
<td>106 ± 4</td>
<td>113 ± 3</td>
</tr>
<tr>
<td>Micro-Hardness (kgf/mm²)</td>
<td>67.6 ± 1.7</td>
<td>71.5 ± 1.3</td>
<td>72.9 ± 1.3</td>
</tr>
</tbody>
</table>

Cortical Histomorphometry

There were no changes in cortical BV, TV, or Ma.V for AEM versus vivarium (Table 2). As expected due to normal growth, BV and TV were similarly and significantly increased by approximately 5% for both vivarium and AEM versus baseline.
Trabecular Microarchitecture

Animal housing environment had a significant effect on trabecular microarchitectural parameters at the proximal tibia, as shown in a representative microCT image (Figure 3). Compared to vivarium, mice housed in AEMs demonstrated greater BV/TV (+44%; Figure 4A) and Conn.D (+144%; Figure 4B). As shown in Table 3, there were also significant differences in SMI (-12%), Tb.N (+11%), and Tb.Sp (-10%) at the proximal tibia for AEM versus vivarium. There was no difference in Tb.Th for AEM versus vivarium. Compared to baseline, AEM-housed animals had 61% greater BV/TV and 193% greater Conn.D. There were no significant differences between baseline and vivarium for any parameters measured at the proximal tibia.

Analysis of trabecular microarchitecture at the proximal humerus revealed that AEM-housed mice had an 18% greater BV/TV than vivarium controls, with a non-significant trend suggesting greater Conn.D (Table 3). These differences were due a decrease in trabecular BV/TV and Conn.D in vivarium-housed mice compared to baseline.

Trabecular Histomorphometry

Trabecular MS/BS was 295% greater for AEM-housed mice versus vivarium (Figure 4C). Osteoid volume, osteoid surface, and osteoid thickness were all significantly lower for AEM-housed mice compared to both vivarium-housed and baseline (Table 4). Eroded surface was 73% lower for AEM-housed versus vivarium.

Trabecular Histology

For AEM-housed mice, tibia trabecular Ob.S/BS was 79% lower than vivarium (Figure 5A), while Oc.S/BS of AEM mice was 65% lower than vivarium (Figure 5B). Compared to baseline, both vivarium and AEM-housed mice had a lower measured osteoblast and osteoclast surface; however, the decrease was greater in magnitude for AEM for both Ob.S/BS (-85% vs. -28%) and Oc.S/BS (-75% vs. -30%).

At the mid-L5 vertebrae, Ob.S/BS was found to be significantly decreased for AEM mice when compared to vivarium (-70%; Figure 5C). Oc.S/BS for AEM mice was 32% lower than their vivarium-housed peers (Figure 5D). There were no differences between baseline and vivarium for either Ob.S/BS or Oc.S/BS. Conversely, AEM-housed animals had a significantly lower Ob.S/BS (-60%) and Oc.S/BS (-30%).

Table 2. Femur cortical histomorphometric parameters. Data was obtained from femur mid-diaphysis cross-sections collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Vivarium and AEM mice received an injection of the bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study. BV=bone volume, TV=tissue volume, Ma.V=medullary volume, Av.Ct.Th=average cortical thickness, Ec=endocortical, Ec.ES=endocortical eroded surface, BS=bone surface. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
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<tbody>
<tr>
<td>BV (mm³)</td>
<td>0.65 ± 0.01</td>
<td>0.68 ± 0.01</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td>TV (mm³)</td>
<td>1.54 ± 0.02</td>
<td>1.60 ± 0.02</td>
<td>1.59 ± 0.01</td>
</tr>
<tr>
<td>Ma.V (mm³)</td>
<td>0.90 ± 0.01</td>
<td>0.91 ± 0.02</td>
<td>0.89 ± 0.01</td>
</tr>
<tr>
<td>Av.Ct.Th (µm)</td>
<td>170 ± 6^A</td>
<td>176 ± 5</td>
<td>194 ± 5</td>
</tr>
<tr>
<td>Ec.ES/BS (%)</td>
<td>-</td>
<td>19.9 ± 2.9</td>
<td>24.5 ± 2.3</td>
</tr>
</tbody>
</table>
Table 3. Trabecular microarchitectural parameters of the tibia and humerus. Tissue was collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. SMI=structure modeling index, Tb.N=trabecular number, Tb.Th=trabecular thickness, Tb.Sp=trabecular separation, BV/TV=trabecular bone volume fraction, Conn.D=trabecular connectivity density. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal Tibia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMI (no units)</td>
<td>3.08 ± 0.06</td>
<td>2.98 ± 0.06</td>
<td>2.63 ± 0.07</td>
</tr>
<tr>
<td>Tb.N (1/mm)</td>
<td>4.07 ± 0.07</td>
<td>4.01 ± 0.09</td>
<td>4.44 ± 0.1</td>
</tr>
<tr>
<td>Tb.Th (mm)</td>
<td>0.036 ± 0.007</td>
<td>0.039 ± 0.006</td>
<td>0.040 ± 0.004</td>
</tr>
<tr>
<td>Tb.Sp (mm)</td>
<td>0.247 ± 0.005</td>
<td>0.252 ± 0.006</td>
<td>0.226 ± 0.006</td>
</tr>
<tr>
<td><strong>Proximal Humerus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>10.4 ± 0.5</td>
<td>8.5 ± 0.4</td>
<td>10.0 ± 0.6</td>
</tr>
<tr>
<td>Conn.D (1/mm³)</td>
<td>80 ± 7V</td>
<td>53 ± 8</td>
<td>73 ± 9</td>
</tr>
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</table>

Table 4. Tibia trabecular histomorphometric parameters. Data was obtained from tibia metaphysis cross-sections collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Vivarium and AEM mice received an injection of the bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study. OV/BV = osteoid volume normalized to bone volume, OS/BS = osteoid surface normalized to bone surface, O.Th = osteoid thickness, ES/BS = eroded surface. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Vivarium</th>
<th>AEM</th>
</tr>
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<tbody>
<tr>
<td>OV/BV (%)</td>
<td>4.6 ± 0.4</td>
<td>4.1 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>OS/BS (%)</td>
<td>19 ± 1</td>
<td>18 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>O.Th (µm)</td>
<td>3.1 ± 0.2</td>
<td>3.0 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>ES/BS (%)</td>
<td>-</td>
<td>4.5 ± 0.6</td>
<td>1.4 ± 0.3</td>
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Mineral Composition

There was no effect of AEM housing on the whole femur dry, organic, or mineral mass (Table 5). Percent mineralization of the femur diaphysis was slightly, but significantly, lower for AEM versus vivarium (-2%), although this was due to a significant increase in diaphysis mineralization for vivarium versus baseline (+2%). Total mineral content of the femur was also greater for vivarium versus baseline (+2%), although the metaphysis mineral content was not. Dry, organic, and mineral mass were similarly and significantly increased for vivarium and AEM versus baseline.
Figure 2. Cortical quantitative histomorphometric parameters. Femur mid-diaphysis cross-sections were collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Vivarium and AEM mice received a single injection of the bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study. Measured parameters included mineralizing surface normalized to bone surface (MS/BS) at both the (A) periosteal surface (Ps.MS/BS) and (B) endocortical surface (Ec.MS/BS). MS/BS was used as an index of bone formation. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05).

Figure 3. Representative images of trabecular microstructure at the proximal tibia. These images illustrate the greater amount of trabecular bone in mice housed in the Animal Enclosure Module (AEM) versus standard Vivarium housing. Images were selected based on group mean for trabecular bone volume fraction (BV/TV). Images were acquired using a µCT20 (Scanco Medical AG; Brüttisellen, Switzerland) with an isotropic voxel size of 9 µm and represent a three dimensional reconstruction of 100 slices (0.9 mm total) of the tibia, immediately distal to the epiphysial plate.
Figure 4. Trabecular microstructural and histomorphometric parameters. Tibias were collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Microstructural parameters assessed by microCT included (A) trabecular bone volume fraction (BV/TV) and (B) trabecular connectivity density (Conn.D). Vivarium and AEM mice received a single injection of the bone label Calcein (20 mg/kg) approximately 22 hours prior to the start of the study. Measured trabecular histomorphometric parameters included (C) trabecular mineralizing surface normalized to bone surface (MS/BS). MS/BS was used as an index of bone formation. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

Table 5. Mineral composition analysis of the femur. Tissue was collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).
Figure 5. Trabecular osteoblast and osteoclast surface parameters. Histological sections were prepared from the trabecular bone of the tibia and L5 lumbar vertebrae collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. We assessed (A) tibia trabecular osteoblast surface normalized to bone surface (Ob.S/BS) and (B) tibia trabecular osteoclast surface normalized to bone surface (Oc.S/BS). These measurements were also made at the L5 vertebrae (C,D). Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

Serum Chemistry

Serum markers of bone formation were lower for AEM-housed mice when compared to vivarium. Osteocalcin levels in AEM-housed mice were 35% lower than their vivarium-housed counterparts (Figure 6A). The effect of housing environment on alkaline phosphatase levels was more modest: AEM-housed mice had alkaline phosphatase levels 25% lower than vivarium-housed mice (Figure 6B). There was no effect of enclosure type on the bone resorption marker TRAP5b (Figure 6C). Alkaline phosphatase was lower for both vivarium and AEM versus baseline, although the magnitude was greater for AEM (-40%) than vivarium (-20%).

Serum calcium was not affected by cage environment (Figure 6D) and there were no differences in serum calcium for either AEM or vivarium versus baseline. AEM-housed mice did have significantly greater serum phosphorus levels (+20%) when compared to vivarium-housed controls (Figure 6E), although this was due to a decrease in phosphorus for vivarium-housed animals versus baseline. Serum phosphorus for vivarium-housed mice was significantly lower than baseline (-19%), while there was no difference between baseline and AEM.
Figure 6. Serum markers of bone turnover. Levels of the serum bone formation marker (A) osteocalcin and (B) alkaline phosphatase were assessed. In addition, we measured the serum bone resorption marker (C) tartrate-resistant acid phosphatase 5b (TRAP5b) as well as serum levels of (D) calcium and (E) phosphorus. Blood was collected from female C57BL/6J mice. Mice were housed for 13 days in standard vivarium cages or flight hardware (Animal Enclosure Module; AEM), with n=12 per group. Baseline control animals (n=12) were sacrificed on the first day of the study. Data are presented as mean ± SEM. Statistical significance between vivarium and AEM is indicated with a hash mark (#; p<0.05); differences between vivarium or AEM and baseline are indicated with V or A, respectively (p<0.05).

DISCUSSION

We have demonstrated that ground-based use of the AEM results in significant, albeit complicated, effects on murine skeletal physiology. With only modest effects on cortical bone, the AEM was found to produce its most dramatic effects in the trabecular bone compartment. At the proximal tibia and humerus, we observed significantly greater trabecular bone volume fraction and connectivity density in AEM-housed mice versus vivarium. Histological analysis at the proximal tibia suggests that increased accumulation of trabecular bone mass was likely due to a cessation of bone resorption, as there was a significantly lower osteoclast surface and eroded surface for AEM mice when compared to vivarium. Previous studies have determined that, beginning at approximately 6 weeks of age, C57BL/6J mice begin to continuously lose trabecular bone mass at multiple sites (Ferguson et al., 2003; Halloran et al., 2002). Given the 8-10 week age of these mice, the maintenance of trabecular bone structure at the proximal tibia and decline in trabecular bone structure at the proximal humerus of vivarium mice is not unexpected. Indeed, the greater trabecular bone mass and changes in microarchitectural parameters in AEM versus vivarium mice at multiple sites suggest that a significant hardware effect is present.

Histological analysis also revealed a decrease in osteoblast surface for AEM-housed animals.
versus vivarium at both the proximal tibia and the L5 vertebrae. Osteoid volume, surface, and thickness at the proximal tibia were all significantly lower for AEM-housed mice. There were also lower endpoint levels of serum osteocalcin and alkaline phosphatase for AEM mice. Taken together, these findings point to an overall decline in osteoblast activity and bone formation (Gundberg et al., 2002; Lumachi et al., 2009). This was not the case, however, for periosteal mineralization surface, which was used as an index of bone formation (Grimston et al., 2011), in femur cortical bone (Figure 2A). We also demonstrated a significant increase in mineralizing surface at the proximal tibia (Figure 4C). In considering the seeming incongruence of these histological and histomorphometric findings, it is also important to consider the methodology underlying the quantitative histomorphometric analyses. By administering a single Calcein label at day 0, one is actually visualizing mineralization sites present at any point over the course study, without being able to resolve fluctuations throughout. Given the results of the microCT and histological assays, it is likely that bone formation was relatively high in AEM-housed mice for the first half of the study, prior to adaptation of their activity and behavior to the new enclosure. This would have increased the relative mineralizing surface and contributed to the increased trabecular bone mass we documented via microCT. However, over the latter half of the study, the anomalous loading declined and bone formation decreased. This is evidenced by the aforementioned decrease in tibia osteoblast surface and decline in serum markers of bone formation. It is important to remember that all of these assays represent the state of bone physiology at the study endpoint only, which limits our interpretation. A single Calcein label was administered in order to limit variability in experimental design as mice in the parallel spaceflight experiment could only be administered a single label prior to launch. In retrospect, double labeling should have been utilized, as it would have provided dynamic indices. This will certainly be considered in future ground-based studies.

There were increased levels of serum phosphorus in AEM-housed animals versus vivarium. Parathyroid hormone (PTH) is one of the primary regulators of both serum calcium and phosphorus homeostasis (Bergwitz and Juppner, 2010), producing increased calcium release from bone breakdown and increased phosphorus excretion via the kidney. The relatively higher phosphorus levels suggest a lower PTH level. There was not, however, a difference in serum calcium. Given the proposed biphasic bone formation response, it may be that serum phosphorus levels were simply lagging behind calcium in returning to baseline following an early increase in bone formation. Additional insight could be gained from longitudinal measurement of serum PTH levels in future studies.

We must also consider that the observed increase in trabecular mineralizing surface, and potentially bone formation, occurred as a result of the reduced activity of bone-resorbing osteoclasts in AEM-housed animals. Normal (i.e., higher) levels of osteoclast-mediated bone resorption in vivarium-housed mice could have removed portions of the Calcein label that were not resorbed in the AEM-housed mice. The presence of these labels, and their measured size, are necessary for the quantification of histomorphometric parameters. These complications highlight one of the inherent limitations of single label histomorphometry (Arnett and Henderson, 1998). Future studies of the AEM should make use of double label histomorphometry over smaller time frames in order to definitively resolve early versus late changes in bone formation.

Differences in cortical bone parameters between AEM- and vivarium-housed mice were modest compared to findings in the trabecular compartment. There were no changes in cortical structural parameters, such as cortical bone volume or cortical bone thickness, for AEM versus vivarium. However, there was significantly lower femur stiffness for AEM-housed mice, accounted for by lower mineral content of the femur diaphysis. Compared to baseline, AEM-housed mice had greater maximum calculated resistance to torsion and cortical thickness – differences not seen in vivarium-housed mice. However, none of these changes were substantial enough to result in functional outcomes in terms of increased bone mass or strength. It is important to note, however, that cortical bone is generally less sensitive to loading and unloading compared
Greater trabecular bone mass and trabecular mineralizing surface and reduced osteoclast surface are the most striking effects of AEM housing. To account for these findings, we propose that the structure of the AEM enclosure itself and the dynamics of group housing likely contribute to altered animal behavior and increased mechanical loading. The AEM has been designed in such a way that it will necessarily modify skeletal loading during normal activity. The wire mesh walls of the AEM module allow the animals to climb on the walls and roof, a behavior that is different from the standard “two dimensional” floor movement found in vivarium enclosures. This wire mesh is critical, however, for mechanisms that remove floating waste in microgravity (Brooks, 1981; Smith et al., 1987). In addition, the wire mesh provides an anchor point for mice during spaceflight, helping to reduce confounding effects of excessive stress on animal physiology (Sonnenfeld, 1999). It is also important to note that while the animals in the AEM are not crowded by NIH standards, they do have 60% less floor area per animal than the standard vivarium enclosures (130 vs. 81 cm²/mouse). The increased density of animals could have led to increased movement (running, jumping, fighting, climbing over other animals, etc.) that could contribute to increased loading and thus increased bone formation and accumulation of bone mass. In addition, it has been proposed that the interaction and companionship afforded by higher density group housing in the AEM may result in less stress for the animals when compared to standard vivarium housing (Morey-Holton et al., 2000). Although not measured here, the stress hormone cortisol and other glucocorticoids are potent activators of bone resorption in both humans and rodents (Gluer et al., 2007; Rehman and Lane, 2003). If glucocorticoid levels were lower in AEM versus vivarium, it may have contributed to the increase in bone mass that was observed. Measurement of stress hormone levels and adrenal mass would be worthwhile additions to future studies involving the AEM.

The record of spaceflight experiments utilizing animal models has yielded variable results with regards to the effects of housing environment on skeletal properties. Indeed, some studies demonstrated rather dramatic skeletal effects of housing type (Lafage-Proust et al., 1998; Morey and Baylink, 1978; Turner et al., 1979; Yagodovsky et al., 1976), while others saw relatively few changes (Bateman et al., 1998; Turner, 1995; Wronski et al., 1998). The results are so variable that one paper describes “arrested bone formation” (Spector et al., 1983), while another concludes there were “normal levels of [trabecular] bone mass and bone formation” (Wronski et al., 1998). Both the exaggerated and blunted effects of spaceflight on the rat skeleton may be an effect of the differing flight and ground control hardware housing conditions. The effects we observed are modest by comparison to the aforementioned studies, yet demonstrate a significant and important influence of the AEM housing environment that should be considered when planning future spaceflight experiments.

A recent study by Blotter and colleagues investigated the effects of ground-based housing of mice in the proposed “Mice in Space” (MIS) system (Blottner et al., 2009), which has not previously flown in space. In this study, investigators compared 25 days of housing in the MIS to individually ventilated cages. The investigators found no difference in bone structure or mineralization between the two groups. However, it is important to note the differences between the MIS and AEM enclosures, as well as variables related to experimental design. Importantly, the MIS system does not have the same wire mesh walls as the AEM. These walls provide an attachment point for the animals during spaceflight, helping to reduce animal stress in microgravity as well as during take off and landing. During ground-based use of the AEM, the mice are able to climbs the walls and roof of the AEM. As mentioned before, this increase in animal activity and loading may have contributed to the observed increase in bone mass. It is also important to note difference in animal age (3-4 months in MIS vs. approximately 2 months in the present study), study duration (25 vs. 13 days), and animal sex (male vs. female) that complicate a direct comparison. The AEM has proven utility on multiple spaceflight missions, although additional investigation of the AEM and novel enclosure systems would be warranted before making a final decision on future use aboard the
ISS or elsewhere.

A broad analysis of current literature performed by Morey-Holton and colleagues indicates that flight hardware housing parameters determined the degree to which spaceflight caused skeletal degradation (Morey-Holton et al., 2000). Specifically, they compared skeletal parameters from rats following nine days of spaceflight. Rats were housed in either AEMs (group housed with six rats in each AEM) or in the Research Animal Housing Facility (RAHF; singly housed rats in each habitat chamber). While individual, RAHF-housed animals experienced substantial declines in bone formation and bone mass, no significant changes were seen in AEM-housed animals. A similar study by Wronski and colleagues found that group housing of rats in AEMs essentially abolishes the effects of spaceflight on the skeletal system (Wronski et al., 1998). Although they did not use the AEM, other studies have demonstrated the confounding effects of group housing of animals on skeletal properties (Vico et al., 1993; Vico et al., 1988). We note that space-flown group housed rats are in relatively crowded conditions and experience substantial loading during normal social activities. It is for this reason that we proposed modifying the AEM to fly mice on STS-108 (Dalton et al., 2003).

Aside from rodent housing in spaceflight hardware, there is also precedent for the physiological effects of housing environment found in research regarding the bone structure of laying hens (Whitehead, 2004). As with rodent housing, the space available to hens and their ability to experience static and activity-associated loading correlated with positive bone mass and strength parameters (Newman and Leeson, 1998). For example, hens housed in cages with perches or in low-level aviary systems had greater leg bone strength, but relatively less of a change in wing bone strength, whereas birds permitted to fly in high-level aviaries showed relatively greater improvements in wing bone strength (Fleming et al., 1994; Knowles and Broom, 1990; Silversides et al., 2012). Positive effects of housing environment on bone quality in these animals are likely due to increases in osteoblast-mediated bone formation, rather than a decrease in bone resorption (Newman and Leeson, 1998; Whitehead, 2004). Much like the present study, this work demonstrates the complicated and perhaps unpredicted consequences of animal housing on bone.

There are many variables to consider when designing a ground-based control for the spaceflight environment, including the spatial distribution of animals, their range of movement, as well as the lack of buoyancy-driven convection. Conventional thinking would suggest that AEM-housed animals on the space shuttle or ISS should be compared to AEM-housed animals on the ground. However, the unique three-dimensional distribution of animals within the AEM during spaceflight, compared to their two-dimensional distribution on the ground, inevitably results in an imperfect comparison; however, this will be the case for any gravity versus microgravity comparison. Despite the limitations of the AEM as a ground-based enclosure, one cannot discount the impressive wealth of information that has been gleaned from spaceflight experiments, including those rodent studies that utilize the AEM. However, considering the complicated effects of the AEM on skeletal parameters presented here and in previous studies, and the logistical hurdles necessary for a complete redesign of flight hardware, it is clear that some adjustments are needed. The best recommendation would be to give the mice a longer acclimatization period to the AEM, or at least the wire mesh cage configuration. Giving the animals two weeks prior to the start of the flight experiment may help to negate some of the early behavioral, activity, and stress changes that occur as the mice adjust to their new environment. In addition, minimizing animal crowding, as much as possible, would be ideal.

In summary, the present study was conducted as a parallel ground-based control of a space shuttle flight experiment aboard STS-108. A comparison of mice housed in AEMs with mice housed in standard vivarium cages revealed a substantial influence of flight hardware on skeletal parameters. The effect of AEM housing was primarily evident in the trabecular bone compartment, with greater bone mass seen at the proximal tibia and humerus. These effects were likely due to a suppression of osteoclast-mediated bone resorption combined with an early increase in osteoblast activity. Although the specific reasons for this effect are not clear, it is reasonable to hypothesize that altered skeletal
loading, with mice living and climbing on the wire-meshed walls and interacting with each other, is a significant contributor. While the AEM will remain an integral part of animal experimentation on the International Space Station, it would be prudent to consider changes to both ground- and space-based utilization of these enclosures in order to minimize these effects.

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