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Original Article

Physical activity engendering loads from diverse directions augments the growing skeleton

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Abstract

Objective: An experiment was conducted to determine if modifying habitual activities to involve mechanical loading from more diverse directions can enhance the growing skeleton. **Methods:** Growing female C57BL/6J mice were housed individually for 3 months in enclosures designed to accentuate either non-linear locomotion (diverse-orientation loading) or linear locomotion (stereotypic-orientation loading) (n=10/cage type). Behavioral assessments were performed daily to quantify cage activity level. Following the experiment, trabecular and cortical bone structure in the humeral head and distal femoral metaphysis were analyzed with μCT. **Results:** Throughout the experiment, groups did not differ in cage activity level. Yet, following the experiment, the proximal humeri of mice that experienced increased diverse-orientation loading had significantly greater trabecular bone volume fraction (p=0.004), greater cortical bone area (p=0.005), greater cortical area fraction (p=0.0007), and thicker cortices (p=0.002). No significant group differences were detected in the distal femoral metaphysis. **Conclusions:** Diverting habitual activities to entail loading from more diverse orientations can augment the growing mouse skeleton. This study suggests that low-intensity activities that produce loads from diverse directions may represent a viable alternative to vigorous, high-impact exercise as a means of benefiting skeletal health during growth.

Keywords: Trabecular Bone, Cortical Bone, Mechanical Loading, Bone Functional Adaptation, Childhood

Introduction

Weight-bearing physical activity during childhood can enhance skeletal structure[1]. Evidence from controlled exercise intervention studies and cross-sectional studies of young athletes suggests that vigorous, high-impact loading is especially effective for augmenting the growing skeleton[2-4]. However, high-impact exercise may not be an appropriate strategy for promoting bone health in all children. High-impact exercise imposes an increased risk of fracture[5,6], as well as other musculoskeletal injuries[7,8]. Therefore, achieving a better understanding of the osteogenic potential of activities involving low- to moderate-impact loading may improve our ability to customize the behavior prescription for bone health in children with various physical abilities and interests.

Bone's anabolic response to loading is known to be modulated by several aspects of the skeleton's mechanical environment, including load distribution[9,10]. Based on controlled external loading experiments with animal models[11], loads originating from irregular directions may be potent stimuli for osteogenesis even if load magnitudes are relatively low[12]. Therefore, low-intensity activities that engender loads from diverse directions may represent a viable alternative to vigorous, high-impact exercise as a means of benefiting bone health[13,14]. Indirect support for a link between load orientation diversity and skeletal structure is provided by cross-sectional studies of athletes showing that individuals engaged in sports in which the hip is loaded in a wide range of directions (e.g., soccer, speed skating, squash) have enhanced femoral neck bone mass and architecture, similar to individuals participating in high-impact sports (e.g., hurdling, volleyball, triple jump,

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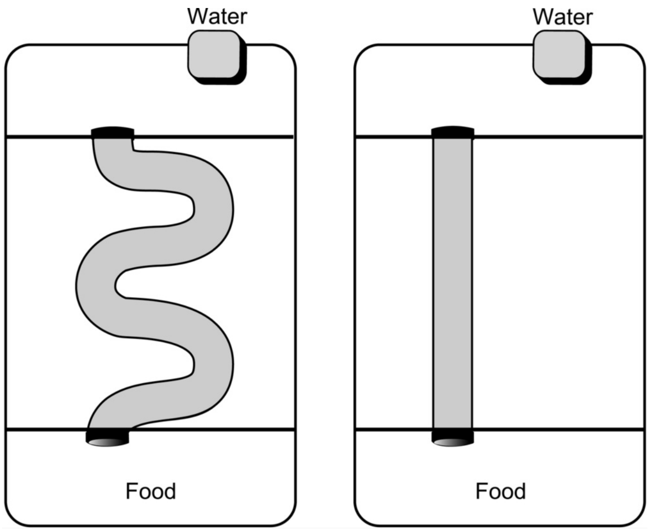

Figure 1. Experimental enclosures designed to emphasize non-linear locomotion (diverse-orientation loading; left) and linear locomotion (stereotypic-orientation loading; right). Enclosures are standard rat cages with modified tunnel apparatuses. Food and water sources were available at opposite ends of the enclosures.

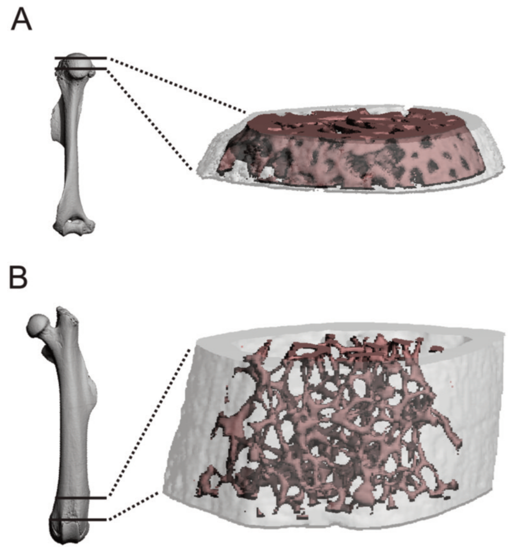

Figure 2. Three-dimensional reconstruction of the volumes of interest in the proximal humerus (**A**) and distal femur (**B**) that were analyzed by μCT, illustrating the separation of trabecular bone from cortical bone.

high jump)[13-15]. However, direct evidence that relatively low-intensity activities producing loads from diverse directions can improve the growing skeleton is currently lacking.

This study examines the effects of emphasizing habitual activities that involve loading from more diverse orientations on skeletal structure in growing mice. Specifically, we test for differences in proximal humeral and distal femoral cortical and trabecular bone morphology between animals housed in custom-designed cages that accentuate either non-linear locomotion (i.e., turning) or linear locomotion[16,17]. Changing direction during mouse quadrupedalism requires eclectic loading of limb joints, especially the shoulder joint since most of the torque to rotate the body is provided by the forelimbs[18]. In contrast, linear locomotion produces loads that are more restricted to a particular plane[19].

Materials and methods

Experimental design

Twenty female C57BL/6J mice were acquired at weaning age (3 weeks) from The Jackson Laboratory (Bar Harbor, ME, USA). The C57BL/6J inbred strain was chosen for study based on previously documented responsiveness of its bone to low-magnitude mechanical signals[20]. At 4 weeks postnatal, animals were housed individually in experimental enclosures (Figure 1) designed to emphasize either non-linear locomotion (diverse-orientation loading) or linear locomotion (unvarying-orientation loading) (n=10 mice per cage type). To limit other activities, particularly climbing, standard wire tops of cages were replaced with flat acrylic crystallite tops, water sources were attached to cage tops with metal sipper tubes protruding vertically downward into cages, and food pellets (standard mouse chow) were placed on cage floors rather than in food dispensers. Animals were maintained under a 12-hr light/dark cycle with access to food and water *ad libitum*. Weekly food consumption was recorded by adding a standardized amount of pellets to cages at the beginning of each week and weighing the remaining pellets at the end of each week. At 16 weeks postnatal, body mass was measured, animals were euthanized by CO_2 inhalation followed by decapitation, and left humeri and femora were extracted and preserved in 70% EtOH. Approval was obtained for all procedures by the Institutional Animal Care and Use Committee.

Behavioral assessments

Daily behavioral assessments were performed throughout the experimental period following a previously described protocol[16,17]. Once or twice daily observations were recorded using an instantaneous focal sampling strategy[21]. A total of 99 behavioral data points were collected for each individual, with observations spread over the entire 24-hr range. Behavioral categories were devised to be self-descriptive and sufficient to represent an overwhelming majority of all observed behaviors[16,17]. Categories were divided into locomotor behaviors (i.e., walk/run, climb, jump) and postural behaviors (i.e., lie, sit, stand). Percentage locomotor behavior was used as a proxy quantitative measure for overall cage activity level. Additional behavioral observations using instantaneous focal sampling with 1-min intervals were performed for 1 hour on multiple days for each individual (n=35 hours per experimental group) in order to verify that rare behaviors in the once or twice daily

	% Lie	% Sit	% Stand	% Postural
Non-linear group	23.5±6.0	37.2±8.2	15.4±4.0	76.1±5.6
Linear group	21.2±9.0	36.4±13.0	18.9±4.9	76.5±9.9
p-value	0.51	0.63	0.094	0.91
	% Walk/Run	**% Climb**	**% Jump**	**% Locomotor**
Non-linear group	16.8±5.4	6.3±4.0	0.9±1.0	23.9±5.6
Linear group	14.2±5.8	8.4±5.5	0.9±1.3	23.5±9.9
p-value	0.33	0.34	0.85	0.91

% lie + % sit + % stand = % postural behavior (i.e., static behavior). % walk/run + % climb + % jump = % locomotor behavior (i.e., cage activity level).

Table 1. Comparison of activity profiles from behavioral assessments.

observations were not underestimated in frequency. Again, the entire 24-hr range was sampled. In order to monitor tunnel use, number of tunnel passes per hour was simultaneously recorded for individuals during the 1-min interval observations.

μCT

A 500-μm-long region of the humeral head was scanned at an isometric voxel size of 10 μm (70 kVp, 114 μA, 200-ms integration time) and a 1100-μm-long region of the distal femoral metaphysis was scanned at an isometric voxel size of 12 μm (55 kVp, 145 μA, 300-ms integration time) using a μCT 40 Scanco Medical scanner (Brüttisellen, Switzerland). The humeral head volume of interest started 100 μm distal to the proximal-most point of the cortical-trabecular bone interface, and the femoral metaphysis volume of interest started 850 μm proximal to the growth plate (Figure 2). Although most studies of trabecular bone mechanoresponsiveness focus on bone tissue in the metaphysis rather than the epiphysis, since epiphyses rarely fracture, the humeral epiphysis was analyzed in the current study because of the low quantity of trabecular bone in the humeral metaphyses of C57BL/6J mice. The femoral neck was also not analyzed due to low trabecular bone quantity. Images were filtered to reduce noise using a constrained 3D Gaussian filter (support=1, σ=0.3) and segmented to extract the bone phase using threshold values that were determined empirically to achieve maximal concordance between raw and thresholded images (trabecular bone: 287.2 mg HA/cm^3; cortical bone: 455 and 922.9 mg HA/cm^3 for the humerus and femur, respectively). Repeatability of this thresholding method is high[22]. Trabecular bone was separated from cortical bone using an automated algorithm[23]. The internal imaging code supplied by the scanner manufacturer was used to calculate bone structural properties, including trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), degree of trabecular anisotropy (DA), total cross-sectional area inside the periosteal envelope (Tt.Ar), cortical bone area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar), and average cortical thickness (Ct.Th)[24].

Statistical analyses

Shapiro-Wilk tests were used to determine if data followed a normal distribution, and Levene's tests were used to assess the equality of group variances. Depending on the results of these tests, differences between the two experimental groups were analyzed with independent samples t-tests, Mann-Whitney U-tests, Welch's t-tests, or generalized Wilcoxon tests. All analyses were carried out using R (version 2.15.3, R Core Development Team 2013). Statistical significance was judged using a 95% criterion ($p<0.05$), and tests were two-tailed. Data are presented as mean ± SD.

Results

Over the course of the experimental period, groups did not differ significantly in overall cage activity level as measured by percent locomotor behavior ($p=0.91$) (Table 1). Animals typically passed through tunnels 20 or more times per hour regardless of the experimental group. Individuals in cages that accentuated linear locomotion traversed tunnels at a higher frequency, but total distance travelled was equalized by the longer distance traveled in a single tunnel pass by individuals with non-linear tunnels – i.e., travel distance per hour in linear tunnels (1454±1164 cm) vs. non-linear tunnels (942±764 cm) was not significantly different ($p=0.18$).

At the end of the experimental period, body mass was not significantly different between the groups ($p=0.25$) (Table 2). Significant group differences were, however, found in proximal humeral bone morphology (Table 2, Figure 3). On average, animals that engaged in increased non-linear locomotion, which generated loads from diverse directions, had 12% greater trabecular bone volume fraction ($p=0.004$), 11% greater cortical bone area ($p=0.005$), 15% higher cortical area fractions ($p=0.0007$), and 12% thicker cortices ($p=0.002$). In contrast, no significant group differences were detected in the distal femoral metaphysis, although animals that engaged in increased non-linear locomotion tended to have higher trabecular and cortical bone quantity (BV/TV and Ct.Ar, respectively), consistent with the pattern detected in the humerus.

	Non-linear group	Linear group	p-value
Body mass (g)	20.5±0.6	21.0±1.2	0.25
Humerus			
Trabecular bone			
BV/TV (%)	34.9±2.9	31.2±2.0	0.004
Tb.N (1/mm)	7.4±0.7	7.2±0.8	0.48
Tb.Th (μm)	60.2±5.5	55.4±5.0	0.089
Tb.Sp (μm)	164±16	169±21	0.55
DA	1.28±0.4	1.28±0.7	0.95
Cortical bone			
Tt.Ar (mm^2)	2.02±0.08	2.08±0.18	0.34
Ct.Ar (mm^2)	0.61±0.05	0.55±0.04	0.005
Ct.Ar/Tt.Ar (%)	30.4±2.5	26.4±1.8	0.0007
Ct.Th (mm)	0.065±0.004	0.058±0.006	0.002
Femur			
Trabecular bone			
BV/TV (%)	5.8±0.8	5.2±1.5	0.29
Tb.N (1/mm)	3.6±0.1	3.6±0.3	0.83
Tb.Th (μm)	39.9±3.5	36.6±2.3	0.051
Tb.Sp (μm)	278±10	278±28	0.99
DA	1.24±0.04	1.24±0.05	0.77
Cortical bone			
Tt.Ar (mm^2)	2.07±0.09	2.02±0.10	0.26
Ct.Ar (mm^2)	0.86±0.02	0.82±0.06	0.099
Ct.Ar/Tt.Ar (%)	41.5±1.3	40.7±1.7	0.25
Ct.Th (mm)	0.15±0.01	0.15±0.02	0.46

Table 2. Comparison of body mass and bone morphometric properties.

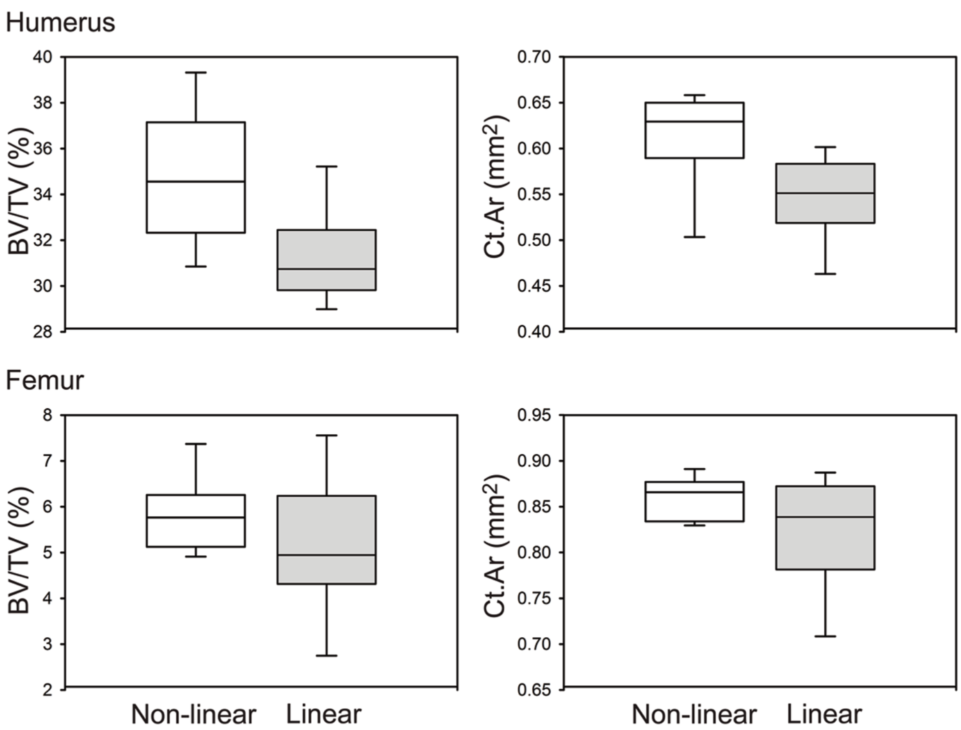

Figure 3. Box plots for proximal humeral (top) and distal femoral (bottom) trabecular bone volume fraction and cortical bone area.

Discussion

This study provides the first direct experimental evidence that modifying habitual activities to entail loading from more diverse directions can augment skeletal structure. Growing mice housed for three months in cages designed to emphasize non-linear locomotion (diverse-orientation loading) were found to have enhanced trabecular and cortical bone in the proximal humerus compared to animals housed in cages that accentuated linear locomotion (stereotypic-orientation loading). Importantly, the observed skeletal differences between the groups were not due to differences in overall cage activity levels (i.e., number of loading events), but rather to the distinct housing conditions that elicited loads from diverse directions during locomotion as opposed to a more restricted pattern of loading. Extrapolating our results from an animal model to humans requires caution, but if such extrapolation is appropriate, then this study suggests that feasible physical activity regimens may be built upon the principles of load orientation diversity for improving skeletal health in children that are unable or unwilling to engage in vigorous, high-impact exercise[13,14]. Examples of physical activities that engender loads from diverse directions, but involve relatively low substrate reaction forces, that are likely to enhance skeletal structure in children include, but are not limited to, dancing, ice-skating, racquet sports, cross-country skiing, and step aerobics[15,25,26]. Although this study focused on the effects of load orientation diversity on the growing skeleton, our results may also be pertinent to the designs of physical activity regimens aimed at stemming bone loss in adults, particularly frail individuals for whom intense exercise imposes a high risk of injury.

That increased non-linear locomotion did not lead to significant structural changes in the distal femoral metaphysis indicates that the effects of diverse-orientation loading vary according to skeletal region. A previous study used the experimental setup employed here to investigate the effects of non-linear locomotion on femoral trabecular bone morphology in growing female BALB/cByJ mice[17]. Consistent with the results reported here for the femur, trabecular bone quantity was found to be unaffected by increased non-linear locomotion. The different response of the humerus and femur to non-linear locomotion is likely related to the functional roles played by the forelimbs and hind limbs when mice change direction during quadrupedal locomotion. When mice turn, most of the torque to rotate the body is provided by the forelimbs[18], which is facilitated by the relatively unrestricted range of movement of the shoulder (a ball-and-socket joint). The hind limbs, in contrast, are primarily responsible for accelerating the rotated body in the new direction[18], which involves motion at the knee (a hinge joint) that occurs approximately in a single plane. Thus, a reasonable hypothesis is that increased non-linear locomotion has a greater effect on the structure of the humeral head than the distal femoral metaphysis because the directions of the loads engendered by turning are more diverse in the shoulder than the knee. However, testing this hypothesis will require additional kinematic and kinetic data on non-linear locomotion in mice. Precise characterization of the mechanical loading environments of the humerus and femur was not technically possible in this study but will be a goal of future research.

Beyond potential implications of this study for public health, this study is also relevant for vertebrate functional morphologists who attempt to infer the locomotor patterns of extinct animals based on bone morphology of fossil remains[27-29]. It has been suggested that trabecular and cortical bone quantity are accurate indicators of overall physical activity levels (i.e., frequency and magnitude of skeletal loading) and that the degree of trabecular anisotropy reflects stereotypy of joint loading and, by extension, locomotor repertoire variability[29-31]. Highly anisotropic trabecular bone is thought to signify a locomotor repertoire that restricts joint mobility to a particular direction, whereas more isotropic trabecular structure is considered to signal locomotor behavior involving greater joint mobility. This presumed relationship between the degree of trabecular anisotropy and load orientation diversity is not supported by the results of this study, in that non-linear locomotion involving diverse-orientation loads was not associated with lower trabecular anisotropy in either the humerus or femur. Moreover, although previous experiments have clearly demonstrated the potential for elevated physical activity levels (e.g., running superimposed onto a normal locomotor repertoire) to increase trabecular and cortical bone quantity[32,33], the results of this study suggest that enhanced bone quantity can be caused by increasing the variation of joint load orientation without a concomitant increase in load magnitude or frequency. Therefore, this study underscores the fact that a particular bone morphological end state can be reached by disparate loading patterns and, likewise, that prudence is necessary when using bone structure to glean information about the locomotor patterns of extinct animals.

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