Journal of Biomechanics 83 (2019) 181–189

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

Single limb immobilization model for bone loss from unloading

Michael A. Friedman [a], Yue Zhang [a], Jennifer S. Wayne [a], Charles R. Farber [b], Henry J. Donahue [a,*]

[a] Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA, United States
[b] Department of Public Health Sciences, University of Virginia, Charlottesville, VA, United States

ARTICLE INFO

Article history:
Accepted 25 November 2018

Keywords:
Unloading
Bone
Model

ABSTRACT

Hindlimb suspension is the most used model for inducing bone loss from unloading but requires a separate ground control group. This control group cannot be used for genetic studies involving outbred mice. In this study, we evaluated a single limb immobilization (SLI) model for inducing bone loss from unloading, with the contralateral limb from the same animal used as a control. Male 10-week old C57Bl/6J mice had one limb immobilized for one, two, or three weeks. Subsequently, an additional group of male 16-week old C57Bl/6J mice had one limb immobilized for three weeks. SLI resulted in decreased tibial trabecular BV/TV, Tb Th, and Tb N compared to contralateral limbs in young mice. Femoral trabecular BV/TV, Tb Th, Tb N, and femoral cortical area fraction were also decreased. Mechanical properties were not affected after three weeks. In adult mice, femoral trabecular BV/TV, Tb Th, and Tb N were decreased. Femoral stiffness, ultimate stress, and Young's modulus were decreased. Bone properties decreased by SLI were also decreased by hindlimb suspension previously. The results suggest SLI can be an effective model for inducing bone loss in growing and adult mice after three weeks of immobilization.

1. Introduction

Mechanical unloading of bones as experienced by astronauts in space and patients on prolonged bed rest leads to decreases in bone volume and bone strength of up to 3% per month (Garland et al., 1992; Lang et al., 2004; LeBlanc et al., 2000). Similar levels of bone loss are seen with aging, resulting in increased risk of fragility fracture. The incidence of fragility fractures is steadily increasing at a rate of 1–3% per year, with an associated cost burden of as much as $20 billion in the United States alone (Cummings and Melton, 2002). Therapies and countermeasures to prevent or attenuate bone loss from unloading are needed to deal with this increasingly burdensome problem.

The underlying mechanisms that lead to bone loss from unloading need to be better understood to develop effective countermeasures. Clues may come from the influence of genetics on the variability in accretion of bone mass and risk of osteoporosis in humans. As much as 80% of the variability in bone mass in the human population may be explained by genetics (Weaver et al., 2016). In relatives of patients with osteoporosis, bone mineral density is lower, and the associated risk of osteoporosis is higher (Seeman et al., 1989; Soroko et al., 1994). Genetics may provide insight to the variable response in humans and may suggest biological mechanisms associated with bone loss, leading to novel therapeutic approaches. However, it remains unclear how genetic diversity affects the response of bone to unloading.

Hindlimb suspension (HLS) is the most widely used and effective animal model for inducing bone loss from unloading (Grimm et al., 2016; Lloyd et al., 2014; Lloyd et al., 2012; Morey-Holton and Globus, 2002). HLS results in decreases in tibial cortical area fraction (−7%) and trabecular bone volume fraction (−25%) as well as loss of femoral stiffness (−14%) and ultimate stress (−10%) in as little as three weeks (Lloyd et al., 2014). However, when testing effects of unloading, HLS or other bilateral unloading models require a ground control group. This control group necessitates the use of inbred mice and is a major limitation for genetic studies. Many of the most powerful mouse gene mapping strategies utilize outbred mice that, unlike mice of inbred strains, are genetically unique and nonreproducible (Svenson et al., 2012). However, using outbred mice would require a different model of unloading that does not use a separate group of mice as a control.

The single limb immobilization (SLI) model can potentially overcome this problem by only unloading one limb and using the contralateral limb from the same animal as a control. Surprisingly, however, this model, by itself, has only been used in the evaluation

* Corresponding author.
E-mail address: hjdonahue@vcu.edu (H.J. Donahue).

https://doi.org/10.1016/j.jbiomech.2018.11.049
0021-9290/

Fig. 1. Mouse with the cast attached to the left limb.

of effects of unloading on muscle and has not been used to evaluate effects on bone in mice (Lang et al., 2012; Speacht et al., 2018). Previous work suggested that one week of SLI results in similar decreases in muscle mass and protein synthesis and similar increases in muscle atrophy gene expression to that of HLS after one week (Lang et al., 2012; Lloyd et al., 2014). Therefore, we hypothesized that three weeks of SLI would result in loss of cortical and trabecular bone volume as well as loss of bone structural and tissue strength in the cast limb, compared to the contralateral control limb. This is the first study to examine the effect of SLI alone on bone in mice.

2. Methods

2.1. Animals

All animal procedures were performed with the approval of the Virginia Commonwealth University Institutional Animal Care and Use Committee. *Young mice*: twenty male, eight-week old C57Bl/6J mice were purchased and given two weeks to acclimate. At ten weeks old, mice were placed in one of two weight-matched groups: cast (n = 11) or sham (n = 9). Three mice from each group were sacrificed after one and two weeks, and the remaining mice were sacrificed after three weeks. Ten-week old mice were used as they were growing and would offer the greatest chance to detect significant differences between cast and sham mice. After discovering that ten-week old mice did indeed lose bone in response to SLI, we then examined the effects of SLI on adult mice. Adult mice have stopped growing and are similar to the age of mice that have been used for HLS and will be used in future casting studies. *Adult mice*: after completing analyses of young mice samples, an additional group of six male, fourteen-week old C57Bl/6J mice were purchased and given two weeks to acclimate. Then at sixteen weeks old, all six mice were placed in the casts. Mice were sacrificed after three weeks in the casts. Cast duration was determined based on the greatest bone loss being detected after three weeks of casting in young mice. Sham mice were not used in adults as the casting protocol was shown to effectively induce bone loss in young mice.

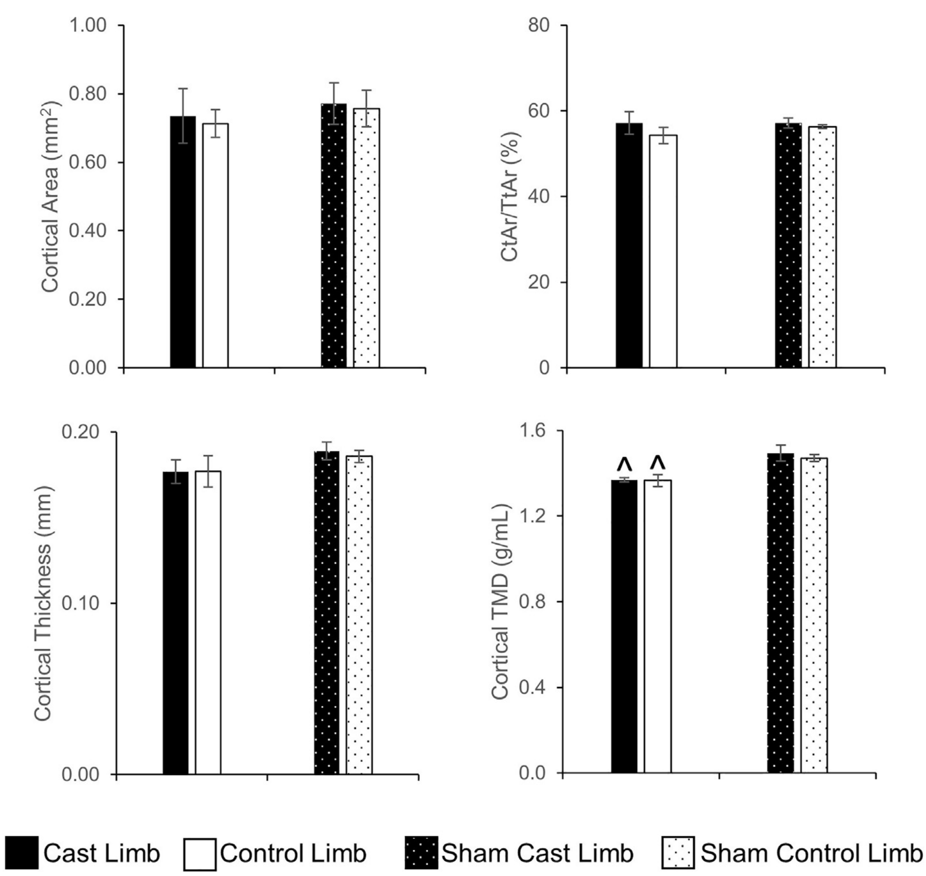

Fig. 2. Tibial cortical bone morphology of the mid-diaphysis in ten-week old mice after three weeks of casting (mean ± SD). n = 3–5 mice. ^p < 0.05 vs. same limb in the sham mice.

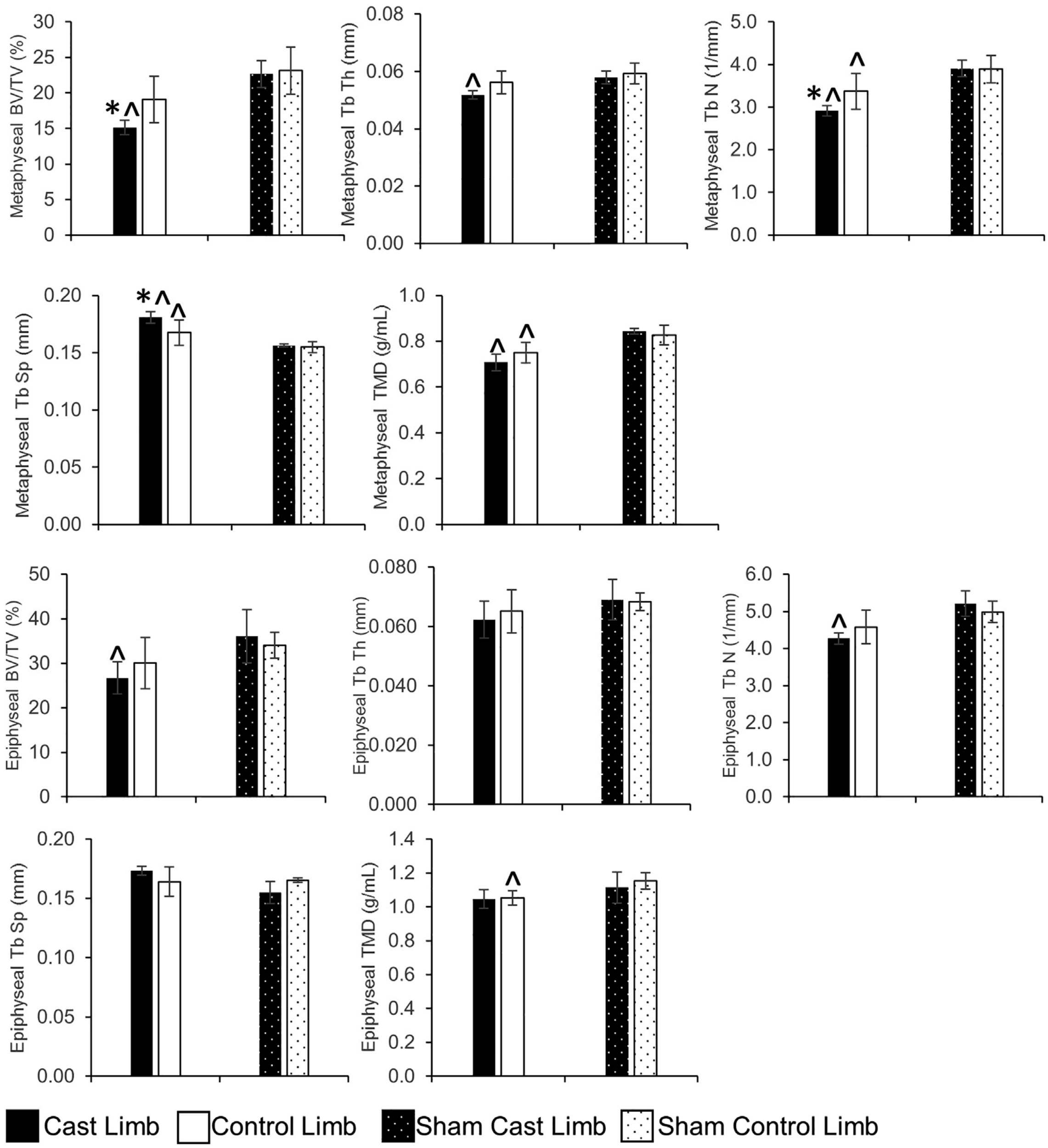

Fig. 3. Proximal tibial trabecular bone morphology in ten-week old mice after three weeks of casting (mean ± SD). n = 3–5 mice. $^{*}p < 0.05$ vs. contralateral control limb. ^$p < 0.05$ vs. same limb in the sham mice.

2.2. Casting protocol

Mice were placed under anesthesia and had the left hind limb shaved. Surgical tape was tightly wrapped around the limb from the paw to the knee. Then a microcentrifuge tube was glued on top of the tape. The tube had the bottom end removed to allow air to flow into it. Right hind limbs were left untreated and used as a contralateral control (Fig. 1) (Lang et al., 2012). Sham mice were only placed under anesthesia and shaved. Mice were single housed and given access to food and water ad libitum. Cast mice were able to freely move around the cages, dragging the immobilized limb. Food consumption was measured in adult mice over a 24-hour period.

2.3. Bone morphology

All tibias and femurs were harvested at sacrifice and stored frozen in calcium buffer. Tibias and femurs from the young mice and femurs from the adult mice were scanned by micro-CT (Skyscan 1173, Bruker microCT). Bones were embedded in 1% agarose gel and scanned at 8.6 μm voxel size, 70kVp, 114 microA, 1.0 mm Al filter, and 1200 ms integration time. Cortical bone morphology was evaluated using a 180-μm slice of the mid-diaphysis to measure cortical area, area fraction (CtAr/TtAr), thickness, and tissue mineral density (TMD). The location was approximately at the center of the mechanical testing region, as described previously (Friedman et al., 2016). Trabecular bone morphology of the

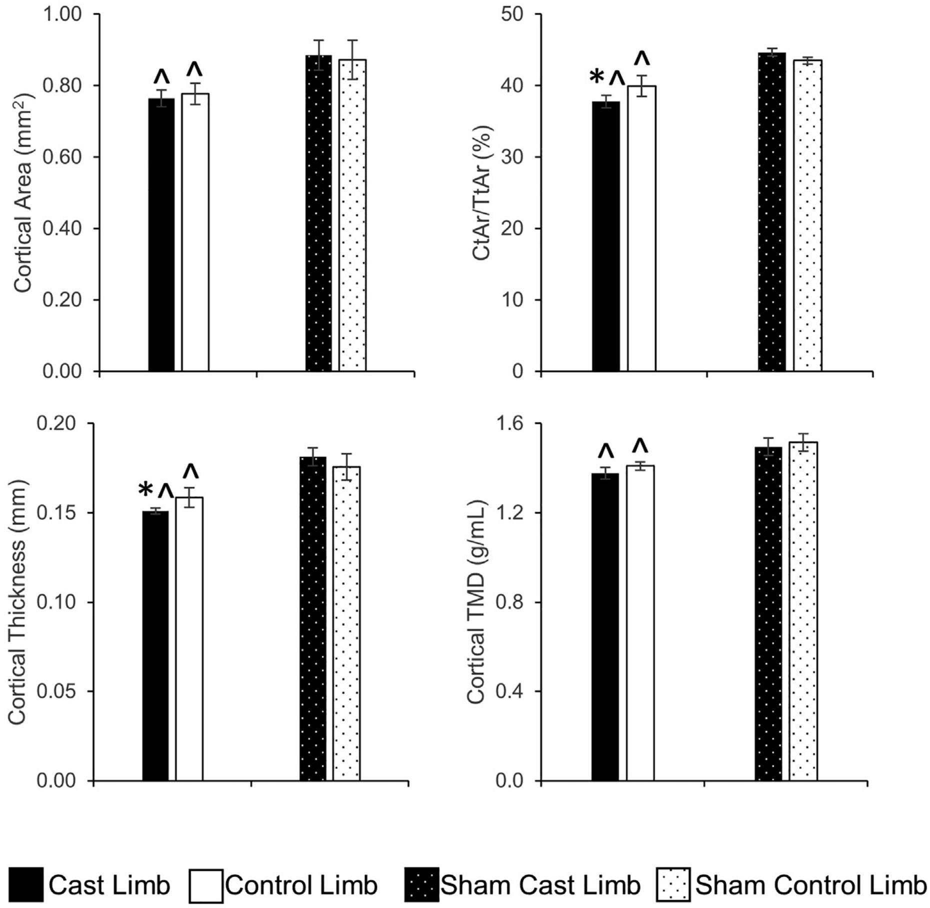

Fig. 4. Femoral cortical bone morphology of the mid-diaphysis in ten-week old mice after three weeks of casting (mean ± SD). n = 3–5 mice. *p < 0.05 vs. contralateral control limb. ^p < 0.05 vs. same limb in the sham mice.

metaphysis was evaluated using a 750-μm slice of the metaphysis taken 200 μm from the growth plate. Computer-traced ROIs were used to measure bone volume fraction (BV/TV), trabecular thickness (Tb Th), trabecular number (Tb N), trabecular separation (Tb Sp), and TMD (Bouxsein et al., 2010). Epiphyseal trabecular bone morphology of the knee was evaluated using a 520-μm slice of the epiphysis immediately adjacent to the growth plate. Freehand-traced ROIs were used to measure trabecular bone properties of the epiphysis.

2.4. Mechanical properties

Mechanical properties were evaluated by three-point bending to failure under displacement control at 1.0 mm/min. Tibias were loaded with the medial side in tension on loading support spans of 10 mm. Femurs were loaded with the anterior side in tension on loading support spans of 8 mm. Stiffness (instantaneous slope of the linear portion of the load-displacement curve at 3.5 N load), yield load (load at loss of 10% of stiffness), ultimate load, yield displacement, ultimate displacement, and energy absorbed were measured (Jepsen et al., 2015). Micro-CT geometry measurements were taken at the location of the fracture site to normalize structural-level properties to give estimates of Young's modulus, yield stress, ultimate stress, yield strain, ultimate strain, and toughness (Turner and Burr, 1993).

2.5. Statistical tests

Two-way ANOVAs and Tukey's tests post-hoc were used to test for significant ($p < 0.05$) differences in young mice after three weeks of casting. Paired t-tests were used to compare casted limbs to contralateral control limbs in adult mice.

3. Results

3.1. Young mice had significant bone loss in the tibia after three weeks

After three weeks, SLI resulted in a significant decrease in tibial cortical TMD in cast and control limbs of cast mice, compared to cast and control limbs of sham mice, respectively (Fig. 2). Casting resulted in significantly decreased tibial metaphyseal BV/TV, Tb Th, Tb N, and TMD, and significantly increased Tb Sp in cast limbs of cast mice, compared to cast limbs of sham mice (Fig. 3). Tibial epiphyseal BV/TV and Tb N were also significantly decreased in cast mice, compared to sham mice. In cast mice, there was a significant difference in metaphyseal BV/TV, Tb N, and Tb Sp between cast and contralateral control limbs.

These effects of casting were significant in the metaphysis after one week and remained after two and three weeks of SLI (Fig. S2). Duration of SLI had no significant effects on metaphyseal trabecu-

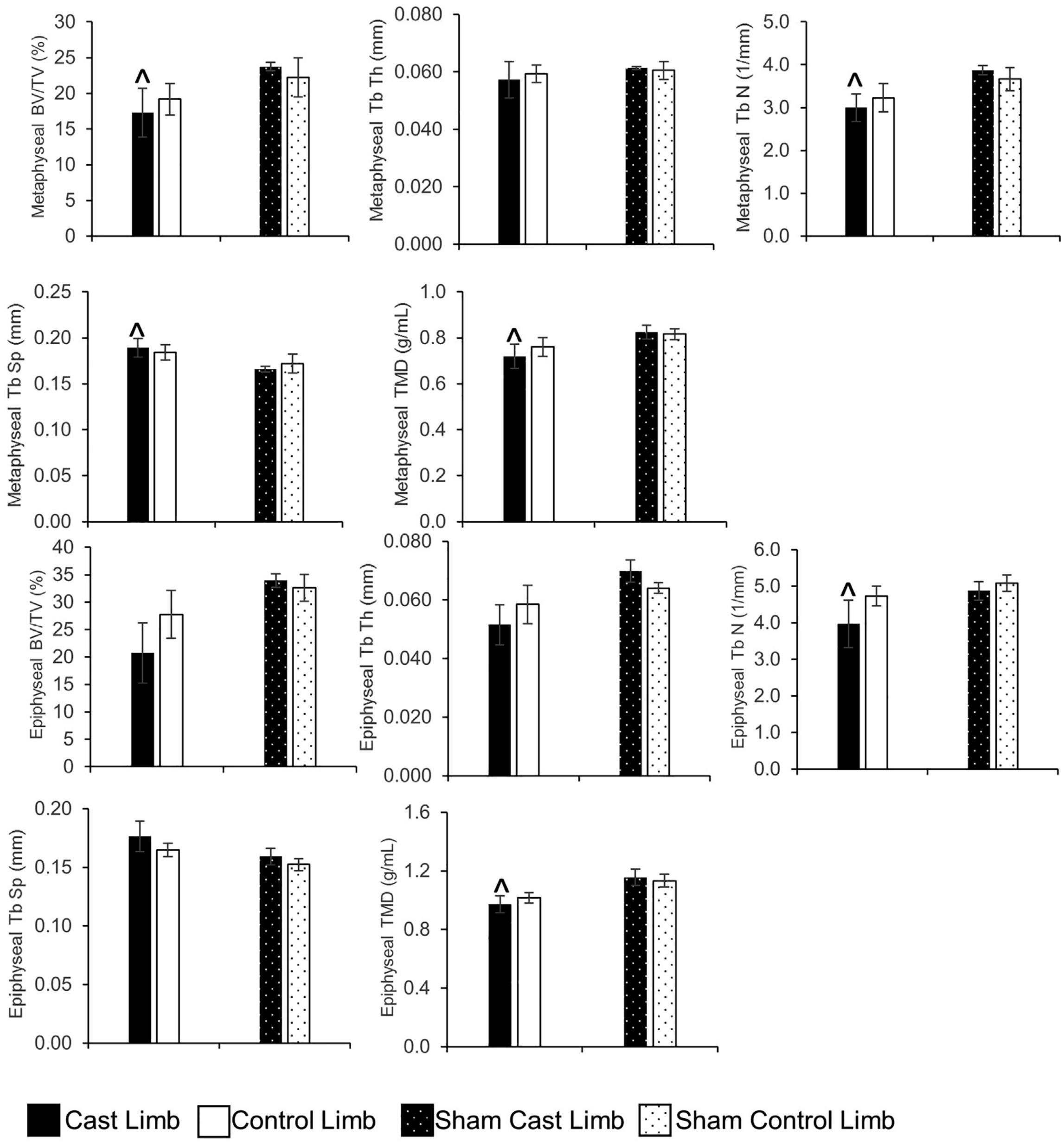

Fig. 5. Distal femoral trabecular bone morphology in ten-week old mice three weeks of casting (mean ± SD). n = 3–5 mice. ^p < 0.05 vs. same limb in the sham mice.

lar bone. In the epiphysis, BV/TV was significantly affected by duration of SLI as there was significantly greater bone loss in cast mice after three weeks than after one week.

3.2. Young mice had significant bone loss in the femur after three weeks

After three weeks, SLI resulted in a significant decrease in femoral cortical area, CtAr/TtAr, thickness, and TMD in cast limbs of cast mice, compared to cast limbs of sham mice (Fig. 4). In cast mice, there was a significant decrease in CtAr/TtAr and thickness between cast and contralateral control limbs. SLI resulted in significantly decreased femoral CtAr/TtAr after two weeks, and cortical thickness was significantly decreased after three weeks (Fig. S3). In cast limbs of cast mice, cortical TMD was significantly decreased after three weeks of SLI, compared to after one week.

Casting resulted in significantly decreased femoral metaphyseal BV/TV, Tb N, and TMD, and significantly increased Tb Sp in cast limbs of cast mice, compared to cast limbs of sham mice (Fig. 5). Femoral epiphyseal Tb N and TMD were also significantly decreased in cast mice. SLI resulted in significantly decreased femoral metaphyseal TMD after two weeks and decreased Tb N after three weeks in cast limbs of cast mice, compared to control limbs of cast mice (Fig. S4). Epiphyseal BV/TV was significantly decreased after two weeks, and epiphyseal Tb Th and Tb N were significantly decreased after three weeks. Tb N was significantly lower after three weeks than after one week. There were no other effects of casting duration on femoral trabecular bone morphology.

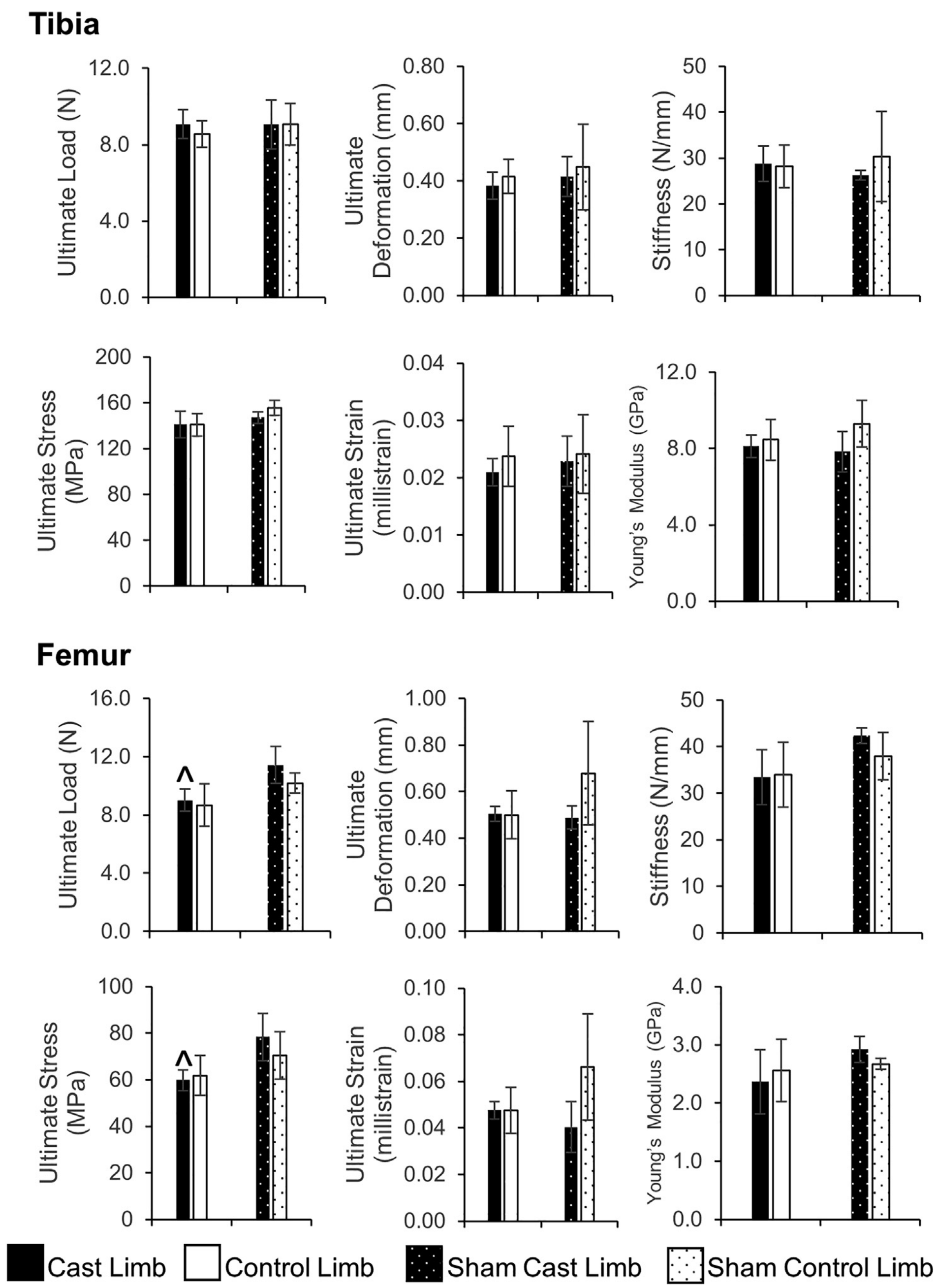

Fig. 6. Tibial and femoral mechanical properties measured by three-point bending to failure in ten-week old mice after three weeks of casting (mean ± SD). n = 3–5 mice. ^p < 0.05 vs. same limb in the sham mice.

SLI resulted in significantly decreased femoral ultimate load and ultimate stress in cast limbs of cast mice, compared to cast limbs of sham mice (Fig. 6). SLI resulted in significantly decreased ultimate stress and Young's modulus in femurs of cast limbs of cast mice after two weeks, compared to control limbs of cast mice (Fig. S5). However, these properties did not remain significantly different after three weeks. No other mechanical properties in the femur or tibia were significantly affected by casting or duration.

Body weight in cast mice did not significantly change from day 1 wt of 24.2 ± 1.2 g (mean ± SD) to after three weeks weight of 24.4 ± 0.9 g (data not shown). Conversely, weight of sham mice significantly increased from day 1 wt of 22.7 ± 1.0 g to after three weeks weight of 24.2 ± 1.2 g ($p < 0.01$).

3.3. Adult mice had significant loss of femoral trabecular bone and bone strength after three weeks

SLI had no significant effects on femoral cortical geometry or metaphyseal trabecular bone after three weeks (Figs. 7 and 8). Epiphyseal trabecular BV/TV, Tb Th, Tb N, and TMD were significantly decreased after three weeks of casting. Unlike young mice, SLI caused significant reductions in mechanical strength in adult mice. Femoral stiffness, Young's Modulus, and ultimate stress were significantly decreased after three weeks of casting (Fig. 9). Body weight significantly decreased ($p < 0.05$) from day 1 wt of 29.7 ± 2.5 g to after three weeks weight of 26.9 ± 2.2 g (data not shown). Food consumption was 4.4 ± 0.9 g per day before casting

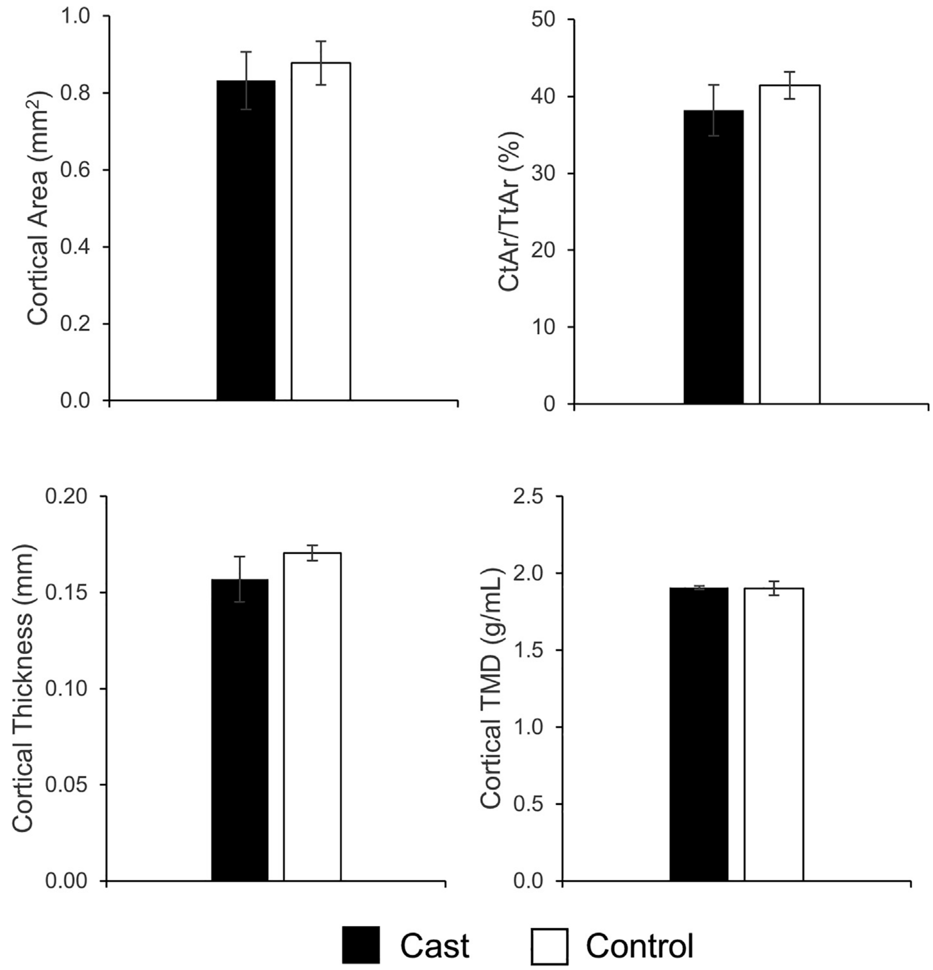

Fig. 7. Femoral cortical bone morphology of the mid-diaphysis in sixteen-week old mice after three weeks of casting (mean ± SD). Cast was the casted left limb and control was the non-casted right limb from cast mice. There were no significant differences. n = 6.

and significantly increased ($p < 0.05$) to 5.2 ± 0.6 g per day after casting (data not shown).

4. Discussion

SLI in young, 10-week old mice and adult, 16-week old mice resulted in tibial and femoral bone loss after three weeks. Unloading from SLI here, and HLS previously, both result in changes in some bone morphology characteristics consistent with bone loss (Lloyd et al., 2014; Speacht et al., 2018). Compared to young mice, SLI was not as effective in causing bone loss in adult mice. However, no sham group was used with adult mice, to compare bone loss from unloading to bone loss from age-related effects (Glatt et al., 2007). Casting was more effective in growing mice, suggesting the differences in bone volume may be a result of preventing bone growth rather than increasing bone resorption.

Bone loss occurred most rapidly in the trabecular bone of the tibia. SLI was most effective in causing bone loss in the metaphysis of the tibia in young mice, with more properties significantly affected by casting. Significant loss of BV/TV appeared in the epiphysis of the tibia and femur after three weeks. The cast covered the limb from the paw to a few millimeters of the femur. Regions of bone furthest from the paw showed the most rapid and greatest magnitude of bone loss. There may have been some loading from the cast being in contact with the ground that resulted in the tibial mid-diaphysis region not having any significant bone loss from SLI.

Despite significant bone loss from SLI in young cast mice, there were no significant effects of casting on bone mechanical properties after three weeks. Since mechanical testing was done by three-point bending of the mid-diaphysis, and most cortical geometry properties of the mid-diaphysis showed no significant effects of SLI, it was unlikely that there would be significant effects on structural-level mechanical properties. In adult mice, there were significant effects of SLI on femoral stiffness, ultimate stress, and Young's modulus despite no significant effects on cortical bone geometry. This suggests there may be differences in bone tissue quality in the adult mice that make their bones more susceptible to fracture after being unloaded by SLI (Bouxsein, 2005; Burr, 2004).

Sham mice were used in addition to SLI in young mice to elucidate whether changes that occurred from casting were independent from age-related changes. Several cortical and trabecular bone geometry properties were significantly lower in cast mice compared to sham mice after three weeks. This could be explained in part by differences in body weight (Selker and Carter, 1989). Sham mice continued to gain weight throughout the study while cast mice body weight remained unchanged. SLI also affected body

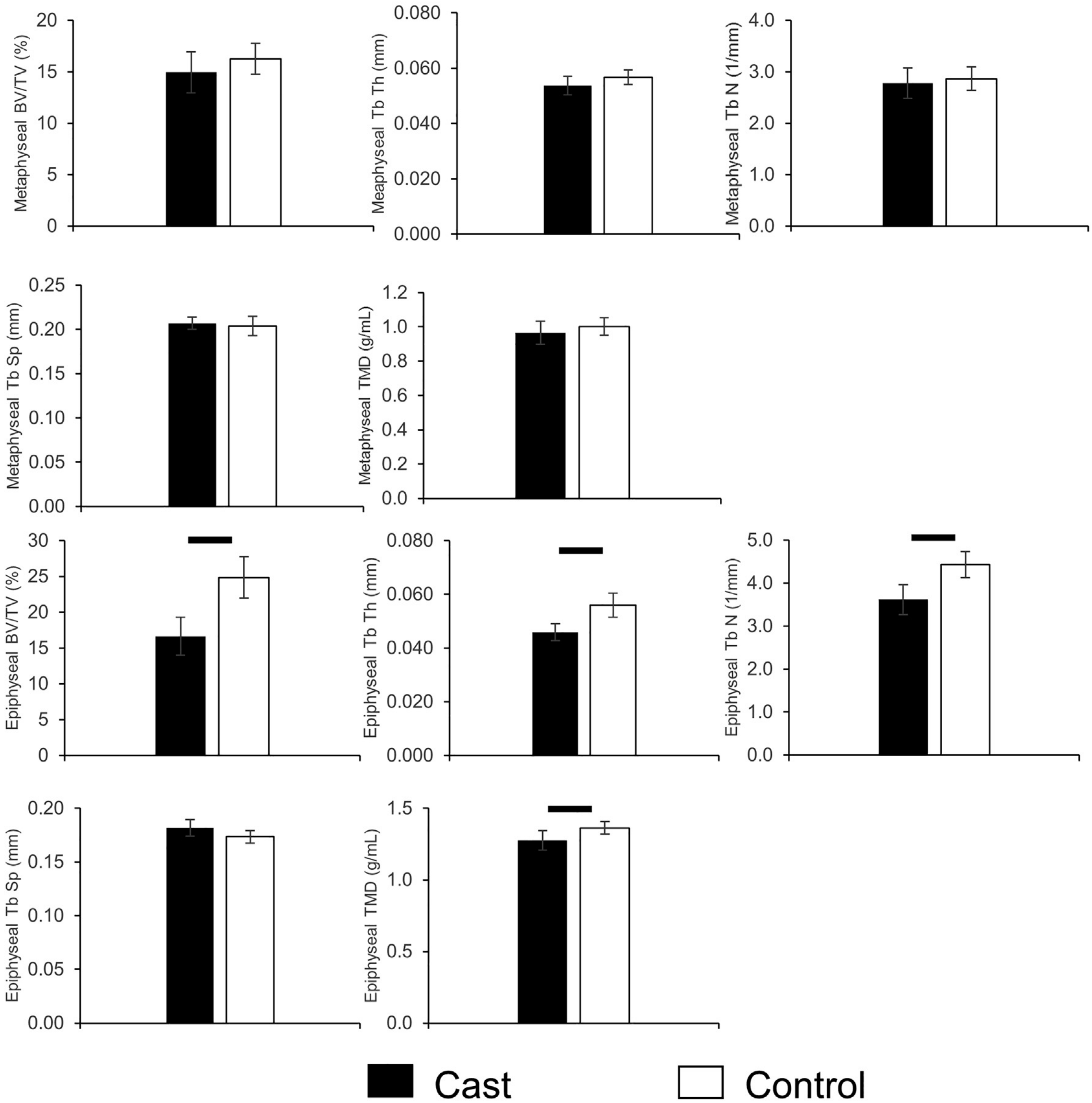

Fig. 8. Distal femoral trabecular bone morphology in sixteen-week old mice after three weeks of casting (mean ± SD). Cast was the casted left limb and control was the non-casted right limb from cast mice. –$p < 0.05$ Cast vs. Control. n = 6.

weight in adult mice as the cast mice suffered a significant decrease after three weeks. Male C57Bl/6 mice are not expected to naturally decrease body weight at any time during their lifespan (Glatt et al., 2007). Food consumption significantly increased after casting in adult mice. There may be other factors involved leading to the decrease in body weight.

The SLI model was effective for inducing bone loss from unloading after three weeks, but further investigation is needed to determine the causes of body weight loss and its effects on bone properties. Decreased body weight can negatively affect bone properties in both casted and untreated limbs. In vivo microCT measurements of bone geometry would give a more accurate measurement of bone loss from SLI as cast limb measurements could be compared to baseline data from the same limb. Additionally, serum markers of bone metabolism could be used to determine if there are any systemic effects of SLI that lead to bone loss from untreated limbs. These measurements could provide a better understanding of the effectiveness of the contralateral untreated limb as a control.

A limitation of this study was the small sample size of young and adult mice used for casting. Since we were developing a model rather than fully examining effects of age on response to casting, we cannot draw strong conclusions on the differences between effects of casting on young mice and adult mice. The purpose of this study was to demonstrate that SLI is an effective model for disuse bone loss and was similar to HLS in this regard. Finding significant bone loss even with a low sample size suggests that SLI can be an effective model.

Bone loss from unloading was effectively produced by SLI in mice of the same age, strain, and gender that were previously used to demonstarte HLS-indcued bone loss. Although the SLI model causes bone loss in the contralateral untreated limb, there were still significant effects of casting on bone geometry and mechanical properties at various sites measured in the tibia and femur. This model can be an effective tool for evaluating bone loss from unloading with genetically unique mice or mice of smaller sample sizes since SLI does not require an additional ground control group.

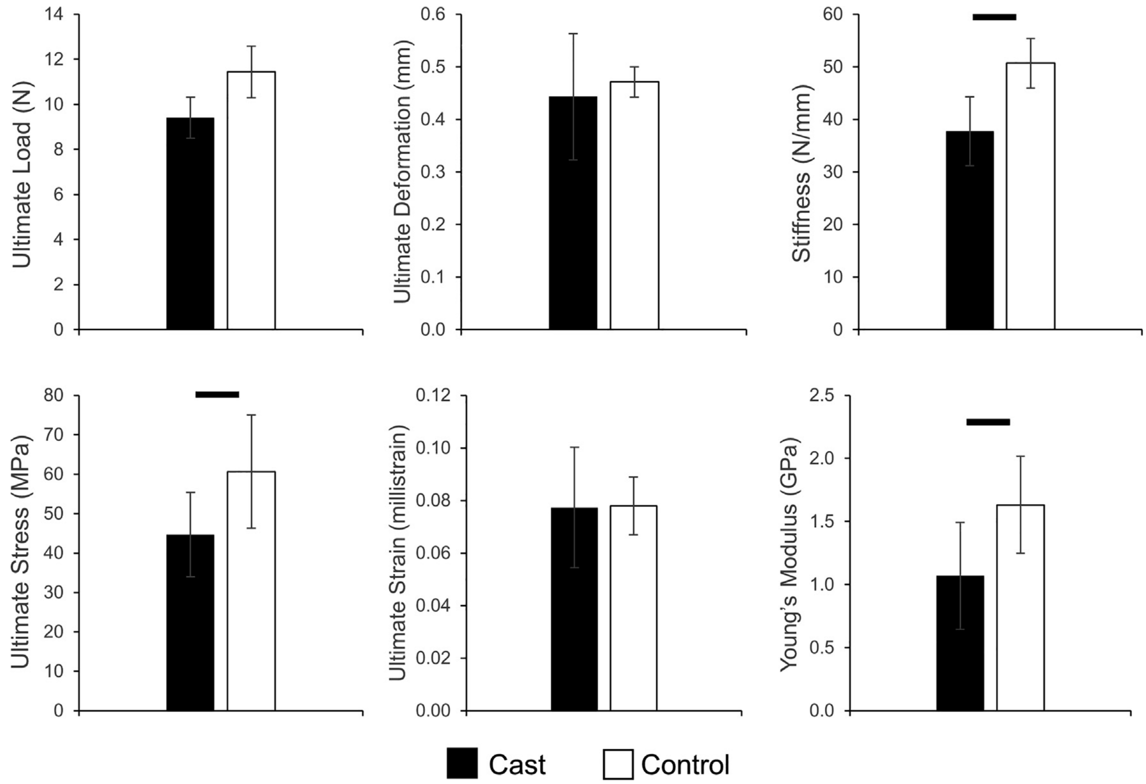

Fig. 9. Femoral mechanical properties measured by three-point bending to failure in sixteen-week old mice after three weeks of casting (mean ± SD). Cast was the casted left limb and control was the non-casted right limb from cast mice. –p < 0.05 Cast vs. Control. n = 6.

Acknowledgments

Virginia Commonwealth University College of Engineering Foundation Research Endowment.

Conflict of interest statement

None of the authors have any conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiomech.2018.11.049.

References

Bouxsein, M.L., 2005. Determinants of skeletal fragility. Best Pract. Res. Clin. Rheumatol. 19, 897–911.
Bouxsein, M.L., Boyd, S.K., Christiansen, B.A., Guldberg, R.E., Jepsen, K.J., Muller, R., 2010. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. J. Bone Miner. Res. 25, 1468–1486.
Burr, D.B., 2004. Bone quality: Understanding what matters. J. Musculoskelet. Neuronal Interact. 4, 184–186.
Cummings, S.R., Melton, L.J., 2002. Epidemiology and outcomes of osteoporotic fractures. Lancet 359, 1761–1767.
Friedman, M.A., Bailey, A.M., Rondon, M.J., McNerny, E.M., Sahar, N.D., Kohn, D.H., 2016. Calcium- and phosphorus-supplemented diet increases bone mass after short-term exercise and increases bone mass and structural strength after long-term exercise in adult mice. PLoS One 11, e0151995.
Garland, D.E., Stewart, C.A., Adkins, R.H., Hu, S.S., Rosen, C., Liotta, F.J., Weinstein, D. A., 1992. Osteoporosis after spinal cord injury. J. Orthop. Res. 10, 371–378.
Glatt, V., Canalis, E., Stadmeyer, L., Bouxsein, M.L., 2007. Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. J. Bone Miner. Res. 22, 1197–1207.
Grimm, D., Grosse, J., Wehland, M., Mann, V., Reseland, J.E., Sundaresan, A., Corydon, T.J., 2016. The impact of microgravity on bone in humans. Bone 87, 44–56.
Jepsen, K.J., Silva, M.J., Vashishth, D., Guo, X.E., van der Meulen, M.C., 2015. Establishing biomechanical mechanisms in mouse models: practical guidelines for systematically evaluating phenotypic changes in the diaphyses of long bones. J. Bone Miner. Res. 30, 951–966.
Lang, S.M., Kazi, A.A., Hong-Brown, L., Lang, C.H., 2012. Delayed recovery of skeletal muscle mass following hindlimb immobilization in mTOR heterozygous mice. PLoS One 7, e38910.
Lang, T., LeBlanc, A., Evans, H., Lu, Y., Genant, H., Yu, A., 2004. Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. J. Bone Miner. Res. 19, 1006–1012.
LeBlanc, A., Schneider, V., Shackelford, L., West, S., Oganov, V., Bakulin, A., Voronin, L., 2000. Bone mineral and lean tissue loss after long duration space flight. J. Musculoskelet. Neuronal Interact. 1, 157–160.
Lloyd, S.A., Lang, C.H., Zhang, Y., Paul, E.M., Laufenberg, L.J., Lewis, G.S., Donahue, H. J., 2014. Interdependence of muscle atrophy and bone loss induced by mechanical unloading. J. Bone Miner. Res. 29, 1118–1130.
Lloyd, S.A., Lewis, G.S., Zhang, Y., Paul, E.M., Donahue, H.J., 2012. Connexin 43 deficiency attenuates loss of trabecular bone and prevents suppression of cortical bone formation during unloading. J. Bone Miner. Res. 27, 2359–2372.
Morey-Holton, E.R., Globus, R.K., 2002. Hindlimb unloading rodent model: technical aspects. J. Appl. Physiol. 1985 (92), 1367–1377.
Seeman, E., Hopper, J.L., Bach, L.A., Cooper, M.E., Parkinson, E., McKay, J., Jerums, G., 1989. Reduced bone mass in daughters of women with osteoporosis. N. Engl. J. Med. 320, 554–558.
Selker, F., Carter, D.R., 1989. Scaling of long bone fracture strength with animal mass. J. Biomech. 22, 1175–1183.
Soroko, S.B., Barrett-Connor, E., Edelstein, S.L., Kritz-Silverstein, D., 1994. Family history of osteoporosis and bone mineral density at the axial skeleton: the Rancho Bernardo Study. J. Bone Miner. Res. 9, 761–769.
Speacht, T.L., Krause, A.R., Steiner, J.L., Lang, C.H., Donahue, H.J., 2018. Combination of hindlimb suspension and immobilization by casting exaggerates sarcopenia by stimulating autophagy but does not worsen osteopenia. Bone 110, 29–37.
Svenson, K.L., Gatti, D.M., Valdar, W., Welsh, C.E., Cheng, R., Chesler, E.J., Palmer, A. A., McMillan, L., Churchill, G.A., 2012. High-resolution genetic mapping using the Mouse Diversity outbred population. Genetics 190, 437–447.
Turner, C.H., Burr, D.B., 1993. Basic biomechanical measurements of bone: a tutorial. Bone 14, 595–608.
Weaver, C.M., Gordon, C.M., Janz, K.F., Kalkwarf, H.J., Lappe, J.M., Lewis, R., O'Karma, M., Wallace, T.C., Zemel, B.S., 2016. The National Osteoporosis Foundation's position statement on peak bone mass development and lifestyle factors: a systematic review and implementation recommendations. Osteoporos. Int. 27, 1281–1386.