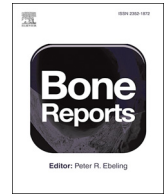




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Treadmill running and targeted tibial loading differentially improve bone mass in mice



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ABSTRACT

Treadmill running and tibial loading are two common modalities used to assess the role of mechanical stimulation on the skeleton preclinically. The primary advantage of treadmill running is its physiological relevance. However, the applied load is complex and multiaxial, with observed results influenced by cardiovascular and musculoskeletal effects. In contrast, with tibial loading, a direct uniaxial load is applied to a single bone, providing the advantage of greater control but with less physiological relevance. Despite the importance and widespread use of both modalities, direct comparisons are lacking. In this study, we compared effects of targeted tibial loading, treadmill running, and their combination on cancellous and cortical architecture in a murine model. We show that tibial loading and treadmill running differentially improve bone mass, with tibial loading resulting in thicker trabeculae and increased cortical mass, and exercise resulting in greater number of trabeculae and no cortical mass-based effects. Combination of the modalities resulted in an additive response. These data suggest that tibial loading and exercise may improve mass differentially.

1. Introduction

Bone is a dynamic structure that can alter its mass and architecture to accommodate a changing environment. Increases in load result in increased bone mass, and conversely, decreases in load result in decreased bone mass. This theory of bone adaptation, referred to as mechanostat, has been well-established in both clinical (Wolman et al., 1991; Warden et al., 2014) and preclinical (Turner, 1998) studies. Within the preclinical realm, studies can often be broadly separated into two groups: 1) exercise-based and 2) external mechanical stimulation.

For exercise studies, animals undergo some form of physical activity, such as running (Iwamoto et al., 1999; Wallace et al., 2007; Wallace et al., 2009), jumping (Umemura et al., 1997; Ju et al., 2008), or swimming (Swissa-Sivan et al., 1990; Hart et al., 2001), causing stimulation of the bone and thereby inducing a bone formation response. The primary advantage of these models is their physiological relevance and translatability. However, this is also the primary disadvantage in that systemic effects are difficult, if not impossible, to fully account for. Loads are multiaxial, and the observed results are influenced by cardiovascular and muscular effects, in addition to the applied mechanical strains engendered on bone. In general, most running studies have shown positive influence on cancellous bone mass and

cortical mechanical integrity, though these results are heavily influenced by sex (Wallace et al., 2007), age (Gardinier et al., 2018), and analysis location (Iwamoto et al., 1999). While these studies are important, the lack of control over the loading profile has made it difficult to assess the effect of specific alterations to loading stimulus or to target specific mechanotransduction pathways.

More recently, external mechanical stimulation protocols have been developed, providing researchers with the advantage of greater control but with less physiological relevance. These protocols apply a direct, predominately uniaxial, load to a single bone or limb, enabling control over the precise stimulus delivered to the bone (Turner et al., 1991; Lee et al., 2002; De Souza et al., 2005). Although a variety of external mechanical stimulation protocols have been utilized, one increasingly popular method is tibial loading, in which the tibia of an anesthetized rodent is placed within a mechanical test device and non-invasively loaded with a cyclic waveform (De Souza et al., 2005). The increased control in these models has enabled researchers to explore types of loads that are anabolic. For example, bone formation is both strain (Sugiyama et al., 2012) and strain rate (Turner et al., 1995; Mosley and Lanyon, 1998) dependent. Mechanistic studies have also been conducted, with targeted loading used to tease out the role of various molecules in mechanotransduction pathways (Saxon et al., 2011; Morse et al., 2014). Despite these advantages, the physiological relevance of

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this model is less clearly defined.

While both types of models engender strain stimulus on bone and induce a bone formation response, previous data in our lab has suggested that exercise and tibial loading may differentially improve bone mass; however, few studies have assessed this question. Results drawn from one type are often used to interpret findings from a different type of loading. The primary goal of this study was to explicitly show some of these differences. Moreover, if mechanisms of bone formation are different, it would imply that the combined effects of exercise and tibial loading should increase bone's response in an additive manner. To test this hypothesis, we compared effects of targeted tibial loading, treadmill running, and their combination on cancellous and cortical architecture in a murine model.

2. Methods

2.1. Experimental overview

All procedures were performed with prior approval from the Indiana University School of Science Institutional Animal Care and Use Committee (IACUC). Male C57Bl/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and separated into two groups: exercise (EX; n = 12) and sedentary control (SED; n = 12). All mice were housed individually to prevent cage fighting which may mask effects of loading (Meakin et al., 2013), but we did not measure the background loading between groups. Beginning at 8 weeks of age, both exercise and sedentary mice underwent compressive loading of the right tibia (details below) for two consecutive days every other week (i.e. Mon/Tues of weeks 1, 3, and 5). The contralateral limb was used as an internal non-loaded control (Sugiyama et al., 2010). On the remaining days of each week and on the alternating weeks, the exercise mice ran on a treadmill (details below). At 14 weeks of age, mice were euthanized and their tibiae stored wrapped in phosphate-buffered saline (PBS)-soaked gauze at -20 °C. This study design resulted in four groups (n = 12/group): 1) no exercise and non-loaded, 2) no exercise and loaded, 3) exercise and non-loaded, and 4) exercise and loaded (Fig. 1).

2.2. In vivo tibial loading

Mice were anesthetized (2% Isoflurane) and their right limb cyclically loaded in compression. Each loading profile consisted of 2 cycles at 4 Hz to 11.9 N, followed by a 1-second rest at 2 N, repeated 110 times for a total of 220 compressive cycles per day. This profile differs from that previously reported from our group (Berman et al., 2015) due to the observation of limping in later cohorts of mice (data unpublished). We found that increasing the frequency to 4 Hz and holding the load at 2 N during the rest period reduced limping. In the current study, limping was not observed. Moreover, the load level is within the range of loads shown to induce a bone formation response (De Souza et al., 2005; Sugiyama et al., 2012; Berman et al., 2015). Note that although

we did not perform histology, we did not observe any signs of woven bone from micro-computed tomography (micro-CT).

2.3. Treadmill running

On exercise days, the exercise group ran on a treadmill (Animal Treadmill: Exer 3/6; Columbus Instruments, Columbus, OH, USA) at a 5° incline for 30 min. Over the first few days of exercise, mice were acclimated to the treadmill, beginning at a rate of 6 m/min and slowly building up to 12 m/min. By the second week, all mice maintained a 12 m/min rate for the duration of the running bout.

2.4. Computed tomography (CT)

Harvested tibiae were scanned by high resolution micro-CT (Skyscan 1172; Bruker, Kontich, Belgium) using the following parameters: 10 μm resolution, 60 kV tube voltage, 167 μA current, 0.7-degree increment angle, and 2-frame averaging. To convert gray-scale images to mineral content, hydroxyapatite calibration phantoms (0.25 and 0.75 g/cm³ CaHA) were also scanned. After reconstruction and rotation using Bruker software (nRecon and DataViewer), regions of interest were selected in the proximal metaphysis, proximal-mid diaphysis, and mid-diaphysis for analysis.

The proximal metaphysis was selected as a 2-mm region of interest, beginning at the distal end of the proximal growth plate and extending distally. Cancellous bone was then automatically segmented from its surrounding cortical shell in CTAn (Bruker) and manually checked for accuracy of segmentation. The region of interest was then analyzed in CTAn to determine bone volume fraction (BV/TV), trabecular thickness (Tb.Th), number (Tb.N), separation (Tb.Sp), bone mineral density (BMD), and tissue mineral density (TMD).

For the proximal-mid and mid-diaphysis locations, a 1-mm region of interest was selected at 37% and 50% of the bone length, respectively, measured from the top of the bone. The cortical shaft was analyzed (Matlab, MathWorks, Inc. Natick, MA) to determine areas (total cross sectional area [Tt.Ar], cortical area [Ct.Ar], and marrow area [Ma.Ar]), cortical thickness (Ct.Th), cortical area fraction (Ct.Ar/Tt.Ar), perimeters (periosteal [Ps.Pm] and endocortical [Ec.Pm]), principal moments of inertia (I_{max} and I_{min}), and tissue mineral density (TMD).

2.5. Statistics

Main effects of exercise and tibial loading were assessed in Prism (v7.03, Graphpad) by repeated measures two-way ANOVA to assess the main effects of loading (within-subject effect), exercise (between-subject effect), and their interaction (p < 0.05). No significant interaction terms were noted for these data and, therefore, no post hoc analyses were performed. Data are presented as mean ± standard deviation.

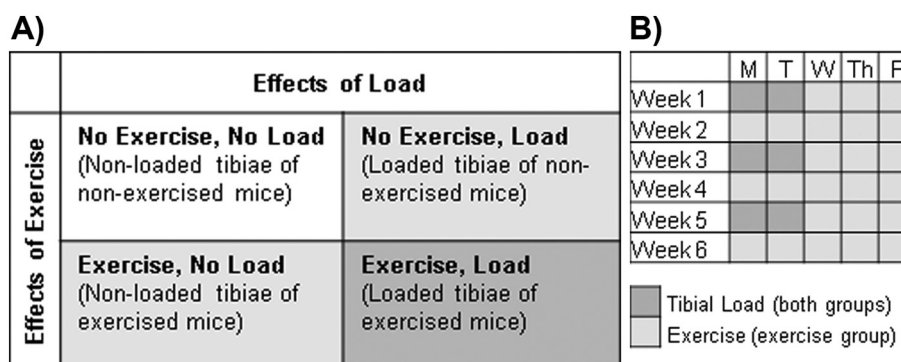


Fig. 1. Experimental overview showing A) groups and B) loading and exercise schedule.

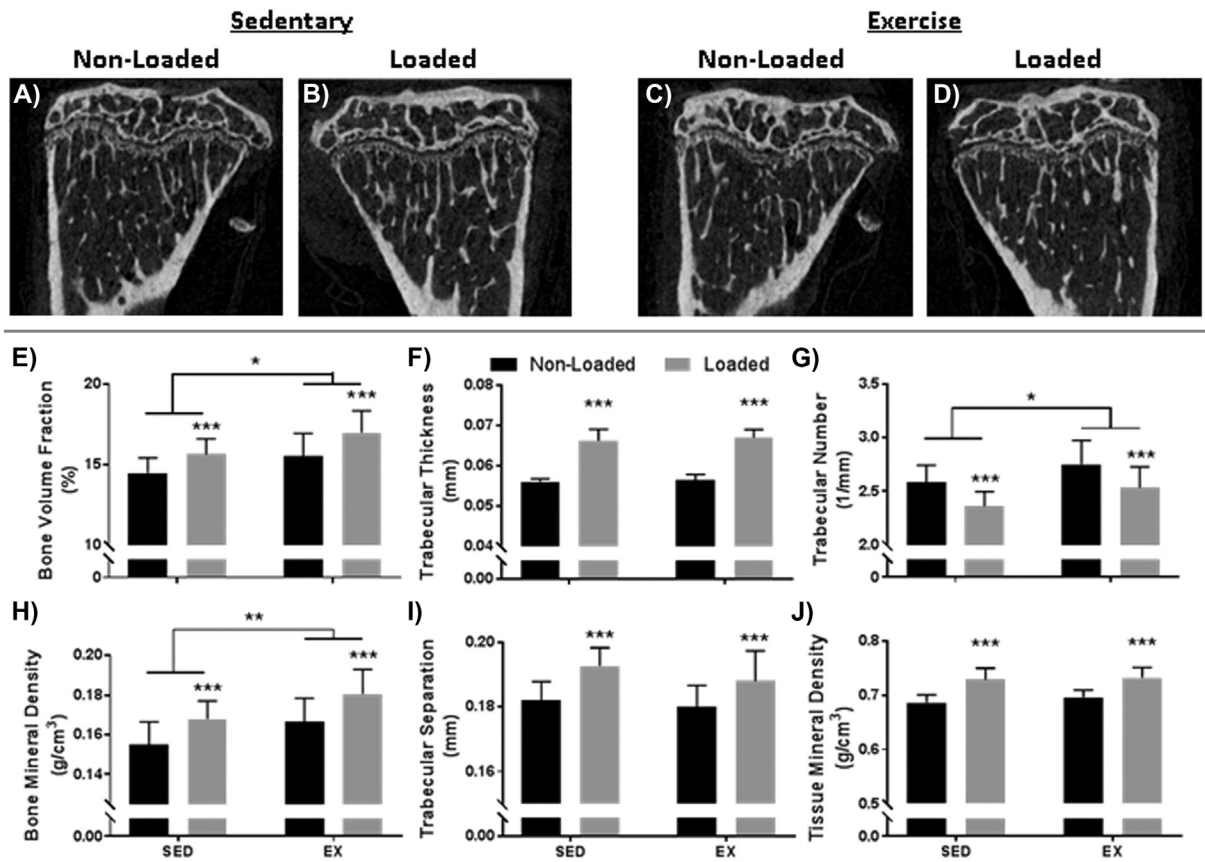


Fig. 2. Cancellous properties within the tibial metaphysis are shown qualitatively (A–D) and quantitatively (E–J). Results indicate improved bone volume fraction (E) and bone mineral density (H) due to both tibial loading and exercise. Combined effects were additive. Tibial loading predominately improved trabecular thickness (F) with detrimental impacts to trabecular separation (I), while exercise predominately improved trabecular number (G). Only tibial loading improved tissue mineral density (J). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Body weight and tibial length

At the beginning of the study, mice were weight-matched into sedentary (23.15 ± 1.56 g) and exercise (23.17 ± 1.56 g) groups. At the end of the study, body weights remained statistically indistinguishable (SED: 26.6 ± 1.4 g, EX: 26.0 ± 1.0 g; $p = ns$). In contrast, tibial length, as measured with calipers at the end of the study, was significantly increased due to loading ($p = 0.02$) but not exercise. Despite its significance, differences in tibial length were modest (SED Non-Loaded: 18.2 ± 0.6 mm, SED Loaded: 18.3 ± 0.5 mm, EX Non-Loaded: 17.9 ± 0.3 , EX Loaded: 18.2 ± 0.4 mm).

3.2. Tibial loading and exercise differentially improve cancellous bone mass

A 2-mm metaphyseal region of interest was analyzed to assess cancellous bone properties (Fig. 2). Results indicated that tibial loading (+7.9%; $p = 0.01$) and exercise (+7.2%; $p < 0.001$) similarly improved bone volume fraction (BV/TV) compared to the non-loaded sedentary bones. Combined effects of the two modalities were additive, showing a 17.2% improvement in bone volume fraction. Similar results were also observed for bone mineral density (BMD), with tibial loading resulting in an 8.3% improvement ($p < 0.001$), exercise resulting in a 7.3% improvement ($p < 0.01$), and their combined effect additive (+16.5%).

Despite similar increases in bone volume fraction and bone mineral density for tibial loading and exercise, the mechanism by which mass was increased varied for the two modalities. Tibial loading primarily

improved trabecular thickness (+18.3%; $p < 0.001$) with detrimental impacts to trabecular number (−8.7%; $p < 0.001$) and separation (+5.8%; $p < 0.001$). In contrast, exercise predominately improved bone mass through greater trabecular number (+6.4%; $p < 0.05$) with modest non-significant changes in trabecular separation and thickness. In combination, additive effects were noted for all three properties (+19.6% for thickness, +3.2% for separation, and −2.0% for number).

Interestingly, although both modalities improved bone mass, only tibial loading significantly impacted tissue mineral density (+6.2%; $p < 0.001$). Exercise modestly improved the value (+1.5%), but results were non-significant.

3.3. Tibial loading, but not exercise, improves cortical bone mass

In contrast to the cancellous region, only tibial loading improved bone mass in the cortical proximal-mid and mid-diaphysis locations. At the proximal-mid location, loading resulted in both periosteal and endocortical expansion, with the loaded bones having a thicker cortex than their non-loaded controls. Similarly, at the mid-diaphysis, loading also resulted in periosteal expansion, as noted by a significantly greater cross-sectional area. Marrow area and endosteal bone surface were reduced, suggesting endocortical contraction at this location. With the exception of periosteal bone surface at the mid-diaphysis location, all structural parameters measured at both the proximal-mid and mid-diaphysis were significantly altered due to loading whereas no properties were significantly changed due to exercise (Fig. 3 and Table 1). Similar to the cancellous region, the proximal-mid location showed increased tissue mineral density with loading. Interestingly, unlike the

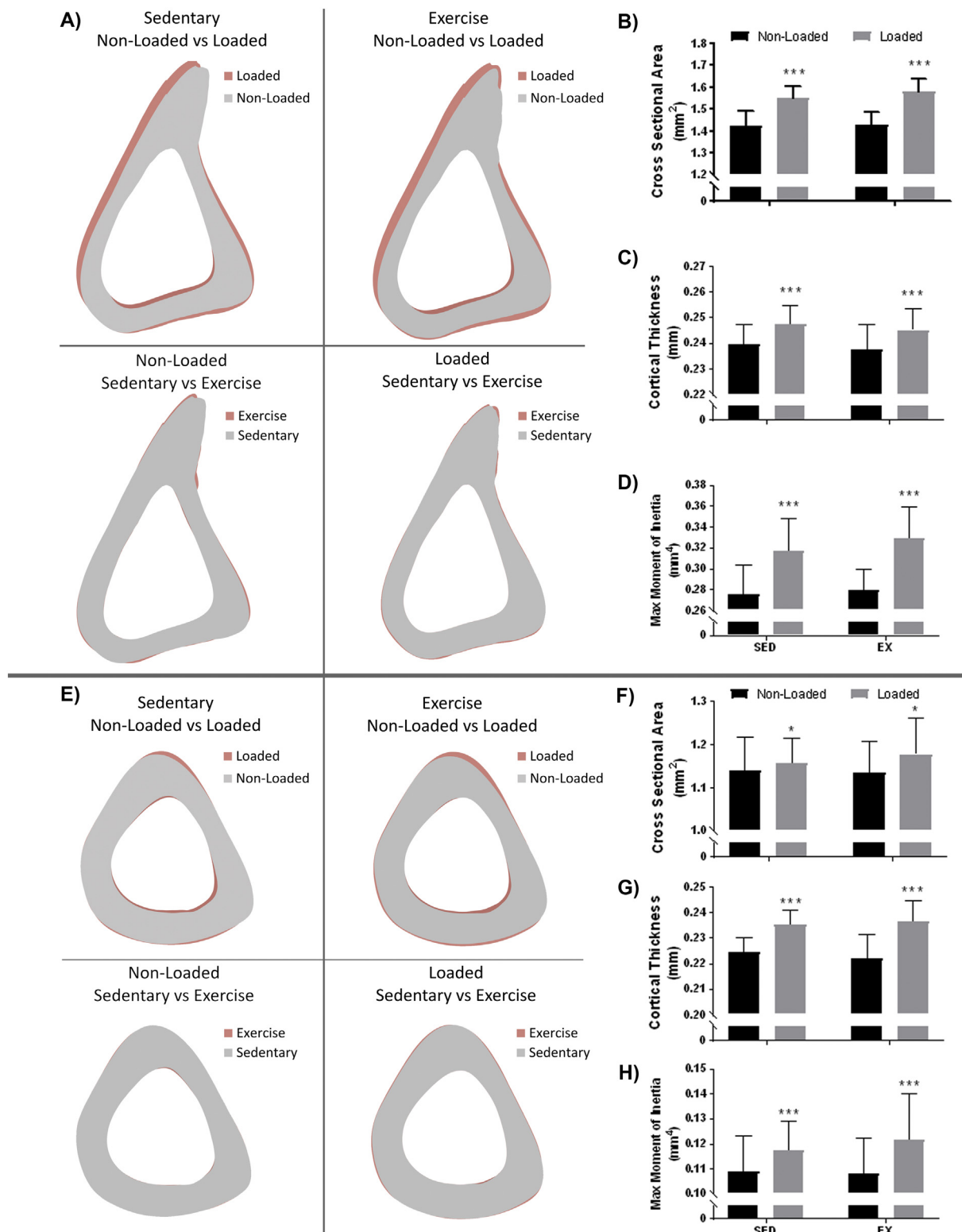


Fig. 3. Cortical analysis at the proximal-mid (A–D) and mid (E–H) locations indicated a strong bone formation response due to loading (A and E, top panels) but not exercise (A and E, bottom panels). These effects can be observed quantitatively through significantly improved cross sectional area (B, F), cortical thickness (C, G), and maximum moment of inertia (D, H) in the loaded limb. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

cancellous and mid-proximal regions, tissue mineral density at the mid-diaphysis was not significantly improved due to loading.

4. Discussion

Tibial loading and exercise are two common modalities used to assess the effects of mechanical stimulation on the skeleton. However, direct comparisons of the two modalities are lacking. In this study, we

Table 1

Quantified cortical parameters from proximal-mid and mid locations indicate improved bone mass due to loading but not exercise. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	Sedentary		Exercise		Two-way ANOVA		
	Non-loaded	Loaded	Non-loaded	Loaded	Exercise	Load	Interaction
Proximal-mid location (37%)							
Cross sectional area (mm^2)	1.421 (0.072)	1.551 (0.052)	1.428 (0.059)	1.581 (0.058)	ns	***	ns
Cortical area (mm^2)	0.839 (0.031)	0.917 (0.029)	0.836 (0.033)	0.921 (0.031)	ns	***	ns
Marrow area (mm^2)	0.582 (0.050)	0.635 (0.039)	0.592 (0.043)	0.660 (0.045)	ns	***	ns
Cortical thickness (mm)	0.240 (0.008)	0.247 (0.007)	0.237 (0.010)	0.245 (0.009)	ns	***	ns
Periosteal bone surface (mm)	6.067 (0.171)	6.164 (0.150)	6.132 (0.173)	6.221 (0.184)	ns	*	ns
Endosteal bone surface (mm)	3.679 (0.149)	3.816 (0.125)	3.700 (0.132)	3.892 (0.150)	ns	***	ns
Max moment of inertia (mm^4)	0.275 (0.028)	0.317 (0.031)	0.279 (0.020)	0.329 (0.031)	ns	***	ns
Min moment of inertia (mm^4)	0.092 (0.012)	0.109 (0.010)	0.093 (0.012)	0.112 (0.010)	ns	***	ns
Tissue mineral density (g/cm^3)	0.780 (0.015)	0.788 (0.012)	0.777 (0.020)	0.783 (0.019)	ns	**	ns
Mid location (50%)							
Cross sectional area (mm^2)	1.139 (0.077)	1.155 (0.061)	1.134 (0.074)	1.176 (0.085)	ns	*	ns
Cortical area (mm^2)	0.693 (0.038)	0.727 (0.030)	0.686 (0.038)	0.738 (0.038)	ns	***	ns
Marrow area (mm^2)	0.445 (0.043)	0.428 (0.035)	0.448 (0.049)	0.439 (0.056)	ns	*	ns
Cortical thickness (mm)	0.224 (0.006)	0.235 (0.006)	0.222 (0.009)	0.236 (0.008)	ns	***	ns
Periosteal bone surface (mm)	4.608 (0.144)	4.639 (0.129)	4.593 (0.144)	4.664 (0.171)	ns	ns	ns
Endosteal bone surface (mm)	3.007 (0.159)	2.937 (0.124)	3.001 (0.174)	2.956 (0.195)	ns	*	ns
Max moment of inertia (mm^4)	0.109 (0.014)	0.117 (0.012)	0.108 (0.014)	0.122 (0.019)	ns	***	ns
Min moment of inertia (mm^4)	0.075 (0.010)	0.077 (0.008)	0.074 (0.008)	0.080 (0.009)	ns	**	ns
Tissue mineral density (g/cm^3)	0.891 (0.017)	0.886 (0.013)	0.883 (0.017)	0.885 (0.021)	ns	ns	ns

show that tibial loading and treadmill running differentially improve bone mass, with tibial loading resulting in thicker trabeculae and increased cortical mass, and treadmill running resulting in greater number of trabeculae and no cortical mass-based effects.

To the authors' knowledge, only one paper to date has utilized both exercise and tibial loading; however, their exercise protocol was mild (3 days/week for 2 weeks) with the aim of supplementing the tibial loading rather than comparing it with tibial loading (Meakin et al., 2015). In addition, they used female mice at 16 weeks of age, compared to our 8 week old male mice. As a result, although the mass-based effects of their tibial loading were similar to our study (e.g. increased bone volume fraction and cortical cross-sectional area), the exercise portion of their results showed no effects, making it difficult to compare the structural changes driven by treadmill running and tibial loading. In contrast, we exercised mice 3–5 times per week for 6 weeks in order to induce an osteogenic response, enabling us to directly compare bone formation due to both exercise and tibial loading.

The tibial loading profile used in this study was similar to profiles reported previously (cyclic, rest-inserted loading) (De Souza et al., 2005; Lynch et al., 2010; Willie et al., 2013), though the presence of rest between cycles has recently been suggested to be unnecessary (Yang et al., 2017). The load, 11.9 N, is within the range of common load levels that have been shown to increase bone volume fraction (De Souza et al., 2005; Lynch et al., 2011; Sugiyama et al., 2012; Berman et al., 2015). Interestingly, despite the plethora of papers utilizing tibial loading, a rarely discussed point is the observation that the increase in cancellous bone mass due to tibial loading is often predominately driven by increases in trabecular thickness rather than trabecular number and spacing (Lynch et al., 2011; Holguin et al., 2013; Weatherholt et al., 2013; Willie et al., 2013; Berman et al., 2015; Yang et al., 2017), though this is not always the case (Lynch et al., 2010; Sugiyama et al., 2010). The current study showed similar results, with a 7.9% increase in bone volume fraction due to an 18.3% increase in trabecular thickness, while the number of trabeculae was significantly decreased. In contrast to tibial loading, the exercise increased bone volume fraction and BMD primarily by increasing trabecular number, suggesting a different mechanism. Based on these results, although tibial loading still can and does provide a wealth of information regarding mechanotransduction, care must be taken in translating observed results due to tibial loading to expected results due to treadmill

running.

One potential reason for these observed effects may be differences in strain (both magnitude and direction) between tibial loading and exercise. Previous studies have shown that low load levels during tibial loading are insufficient to induce a bone formation response (De Souza et al., 2005; Sugiyama et al., 2012; Berman et al., 2015). For that reason, we chose a higher load value that was within the range of loads shown to induce a formation response. However, by doing so, we also presumably selected a strain level that was much higher than is typically observed during treadmill running. For example, in 12 week old mice, walking was found to engender approximately 200 $\mu\epsilon$ of tension, but in the same study, the authors found that approximately 1500 $\mu\epsilon$ (i.e. 10 N) was required to engender a bone formation response during tibial loading (De Souza et al., 2005). Thus, the 11.9 N load that we used in the current study likely engenders much higher strain than treadmill running.

Another important difference to note is that, during tibial loading, the loading direction is more uniform. Although the natural curvature of the tibia causes eccentric loading (i.e. bending) that leads to regions of compression and tension (Yang et al., 2014), the load is still applied in a more uniform, predominately uniaxial, direction without the influence of off-axis muscle and ground reaction forces. This may enable increased alignment of bone formation, causing greater trabecular thickness. Given these differences, it is unlikely that there would be a perfect profile that would mimic the response observed due to exercise.

Within the cortex, differences in bone formation were also noted, with tibial loading causing improved cortical area and exercise having no mass-based effect. Both results are similar to those observed previously (Kohn et al., 2009; Berman et al., 2015; Hammond et al., 2016). However, it should be noted that this study only explored microstructural mass-based changes, and did not assess histology or quality-based changes to the tissue. Thus, the lack of bone mass response observed in the exercised mice does not imply that the matrix is unaffected. Previous work has demonstrated that treadmill running (both with and without changes in mass) can improve aspects of bone quality, predominately the collagen components (Wallace et al., 2007; Kohn et al., 2009; Wallace et al., 2009; Hammond and Wallace, 2015). Recent work has corroborated that these changes in tissue quality can have positive effects on mechanical properties associated with bone ductility (Gardinier et al., 2018) and toughness (Hammond et al., 2016).

Similarly, tissue-level changes have been observed due to tibial loading, with short-term loading altering the mineralization of the matrix in both an age-dependent (Aido et al., 2015) and bone site-specific manner (Bergstrom et al., 2017), leading to improvements in tissue-level mechanics (Berman et al., 2015).

In summary, we have shown that, in the cancellous region, tibial loading led to greater trabecular thickness while exercise led to greater number. Both improved bone volume fraction, with their combined effects resulting in an additive bone mass response. In the cortical region, only tibial loading resulted in increased bone mass, though that does not preclude the presence of non-mass based effects. These data suggest that tibial loading and exercise may improve mass differentially.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Declarations of interest

None.

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