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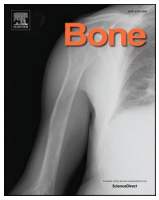

Full Length Article

Regional modular responses in different bone compartments to the anabolic effect of PTH (1-34) and axial loading in mice

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ABSTRACT

Beneficial effects of intermittent parathyroid hormone (PTH) on bone mass and architecture are described to either simply add to, or to synergise with those of mechanical loading. We evaluate whether interaction with in vivo loading is reinforced by PTH dosing regimen and exhibits compartment-specific sensitivities. Female 12-week-old C57Bl6 mice received daily (7/7) or interrupted 5 day/week (5/7) PTH for 3 weeks (two vehicle groups). All mice had six loading episodes (12N) applied to right tibia (left, non-loaded) for the last 2 weeks. Micro-CT scans were used to evaluate mass and architecture in almost the entire cortical and proximal trabecular regions. Epiphyseal cortical, trabecular and marrow space volumes, and bony growth-plate bridge incidence were evaluated. Statistical analyses employed a linear mixed-effects model at each percentile and 2-way ANOVA with post-hoc test for epiphyses and bridging. We found that daily PTH enhances cortical mass and modifies shape along almost the entire tibia and that these effects are partly mitigated by brief interruption in treatment. Mechanical loading alone augments cortical mass and modifies shape but only in a region proximal to the tibiofibular junction. The effect of combining load and daily PTH dosing is solely additive for cortical bone mass with no significant load: PTH interaction, but exhibits clear synergy with interrupted PTH treatment. Daily, not interrupted PTH stimulates trabecular bone gains, yet load:PTH interaction is present at limited regions with both daily and interrupted treatment. PTH treatment, but not loading, modifies epiphyseal bone but, in contrast, only loading modifies bridge number and areal density. Our findings demonstrate impressive local effects on tibial mass and shape of combined loading and PTH that are sensitive to dosing regimen and exert their effects modularly. These findings emphasise a need to clarify PTH dosing regimens and that advantages could be accrued by aligning treatment accordingly to patient requirements and life-style.

1. Introduction

Osteoporosis is the most common worldwide skeletal disease [1,2] and its prevalence is predicted to increase significantly in the next decade [1]. Treatments that seek to enhance bone mass or prevent bone loss to mitigate fracture risk will help to circumvent the development of associated morbidity and mortality [1]. The identification of a new therapeutic option for osteoporosis at the *interface* of pharmacotherapy and bone mechanoadaptation has recently been emphasised, with the osteogenic effects of parathyroid hormone (PTH) administration being augmented further by its interaction with mechanical loading [3–6]. Despite these advances and the obvious benefit that may be accrued by refinement at this interface, there remains ambiguity regarding the scope for optimising the impact of such pharmacological treatments via lifestyle measures involving increases in load-bearing.

The calcium homeostasis achieved by PTH led to early studies that sought an osteogenic treatment for osteoporosis using PTH administered by infusion at a constant rate. This protocol, however, endorsed a catabolic effect for PTH [7,8]. Later, it was found that PTH (1–34) requires an 'anabolic window' to be osteogenic; only intermittently administered PTH resulted in the desired increases in bone mass. The osteogenic effect of intermittent PTH arises via several pathways that ultimately increase the number of osteoblasts; this led to its approval as the first anabolic treatment for osteoporosis [7,8]. Pre-clinical studies have shown that PTH is able to reverse the effects of ovariectomy, with sustained effects evident after 6 months in monkeys, and for 2 weeks after withdrawal in mice [4,9,10]. Intermittent PTH (1–34) treatment has also been shown to increase epiphyseal bone volume, which has

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been speculated to exert beneficial indirect effects on joint cartilage in mouse models of osteoarthritis [11]. These data indicate that intermittent PTH is osteogenic in cortical, trabecular and epiphyseal bone compartments.

The effects of applied mechanical load have been researched in the mouse tibia for the past two decades. It is a highly utilised model in which osteogenic effects of bone loading can be explored [12–14]. The effects of applied mechanical loads are known to be influenced by sex, age and by the precise composition of the applied loading protocol (magnitude, episodes and frequency) [12,15,16]. Mechanical load-induced bone formation is most pronounced in young, growing male mice and the levels of strain engendered by applied loads are greater in the tibiae of ageing female mice, in which the osteogenic effects of loading are almost complete lacking [13,16]. These responses to mechanical load are not uniform, with the tibia having been shown to behave 'modularly' to achieve organ level adaptation, where the scale of this mechanoadaptive response differs across distinct bone compartments [14]. For example, it has been found that the load-related increases (after six episodes over two weeks) in cortical bone formation are mostly concentrated to the proximal 10–60 % of the tibia length, and are largely absent distal to the tibiofibular junction [12,14]. Recently, the clinical impact of such in vivo experiments in murine loading models was emphasised by a study in which post-menopausal women who 'hopped' on one leg to achieve unilateral axial loading, presented an increased trabecular mass in their loaded compared to contralateral tibia, which served as an internal control [3].

The possibility that these osteogenic effects of PTH and mechanical loading may interact has been elegantly studied in normal [17] and osteoporotic adult (>12 week-old) mice following ovariectomy [4,18,19], and in ageing (19 month-old) female mice [5]. The intermittent PTH dosing regimens used in these studies, however, varied in the concentration of PTH administered (25–100 μg/kg) and in its application frequency, with both daily and *interrupted* (5 days on/2 days off) dosing used. These studies point to variation in the extent of interaction between PTH and mechanical load, with reports of either additive or synergistic interaction in different tibial bone compartments [5,18,19]. Using an interrupted PTH dosing regime in ovariectomised adult mice, Roberts et al. [4] described a beneficial additive load:PTH interaction that was most marked in mid-diaphyseal and postero-lateral tibial cortical bone, where predicted mechanical strains are highest, but with no co-treatment benefit in the tibial trabecular bone regions, which show greatest sensitivity to loading alone [4]. Other studies using this same PTH dosing and a 2 week-long 12 N, 3 day/week loading protocol in ovariectomised mice reinforced this solely additive load:PTH interaction in the proximal tibia that resulted in higher periosteal apposition, bone mineral content, and also increased stiffness and strength [18,19]. Intriguingly, similar delivery of PTH using an interrupted regime coupled to a prolonged (5 week-long), 5 day/week loading protocol in intact mice found that PTH and load interact additively in trabecular, and synergistically at the periosteal surface to increase tibial cortical bone mass [17]. Contrastingly, however, the likelihood that these load:PTH interactions are synergistic in intact mice has been questioned in aged female mice, where daily PTH treatment (7/7) interacted only additively with loading to increase periosteal enclosed area [5].

These data suggest that the load:PTH interaction can be optimised for greater osteogenic impact. The lack of studies directly comparing differing dosing regimens, or which simultaneously explore the scale of osteogenic responses to PTH and applied mechanical loading across different bone compartments and regions, has meant that the origins of the synergistic load:PTH interaction remains, however, elusive [4,5,17–19]. With view to defining whether intermittent PTH pharmacotherapy might be better optimised through its combination with mechanical loading, we have sought to clarify whether the load:PTH interaction is sensitive to daily (7/7, PTH 7 days/week) or interrupted (5/7, PTH for 5 consecutive days followed by 2 days without dosing) PTH dosing. We also seek to establish whether the scope for applied mechanical loading to interact with PTH: i) exhibits modular targeting of specific bone types, or ii) is regionalized to particular bone sites.

2. Materials and methods

2.1. Animals

Thirty-nine 11/13 week-old female C57Bl6 mice (Charles River or Harlan) were housed in groups of five in polypropylene cages and subjected to 12 h light/dark cycle, with room temperature maintained between 19 and 23 °C and fed ad libitum with a maintenance mice diet and water.

2.2. Groups and doses

Mice were weight-matched to form four similar groups ($n = 9/10$) that received either an uninterrupted, daily (7/7) or interrupted (5 days on/2 days off) regime of intraperitoneal injections of 40 μg/kg PTH [11,18,20] [8 μg/ml] or matching daily or interrupted injections of vehicle alone (comprising 2 control groups; Fig. 1).

2.3. Formulations/preparations

The vehicle comprised 700 μl of 1 % acetic acid and 1400 μl of 2 % heat-inactivated mouse serum in 67.9 ml Hanks buffered saline solution. The PTH (1–34) (Cat #: P3796 Source: Sigma) formulation was prepared by adding 2.0 ml of vehicle to the vial containing 500 μg PTH. This 'stock' 250 μg/ml PTH solution was subsequently diluted with a further 60.5 ml vehicle to produce 62.5 ml of PTH solution at a concentration of 8 μg/ml. All PTH and vehicle injections were delivered in the morning to all groups, and after the end of the loading procedure of each mouse on days when the tibia was loaded.

2.4. Loading protocol

After one (the first) week of dosing, the right hind limb of each mouse in all four groups was subjected to 12 N dynamic axial loading (0.1 s trapezoidal-shaped pulse period [0.025 s fall time, 0.05 s hold, 0.025 s rise time, 9.9 s rest: 40 cycles] under isoflurane-induced anaesthesia, for 3 days each week, for 2 weeks; (the left hindlimb served as a non-loaded contralateral control) [12]. Anaesthesia was induced in an appropriate chamber with isoflurane diluted in a continuous flow of 100 % oxygen, the delivery of which was calibrated, vaporized and maintained through a face mask.

2.5. Endpoint

Twenty-one days after the first injection, and three days after receiving their final episode of loading, the mice were euthanized using a rising concentration of CO_2, followed by confirmatory cervical dislocation. Right and left hind limbs were skinned, removed and fixed in 4 % PFA for 24 h, washed in distilled water and then stored in 70 % EtOH at room temperature until scanning.

2.6. Micro-CT images

Right and left tibiae from each mouse were scanned in a Bruker Skyscan 1172 desktop micro-CT with the following configuration: Image pixel size 4.98 μm, source voltage 49KV, source current 200 μA, 0.5 mm aluminium filter, rotation step 0.600 and image format tiff. Raw images were reconstructed in NRecon® and reoriented in Dataviewer® software.

2.7. Cortical and trabecular bone segmentation

Micro-CT image segmentation was performed using CTAn® (Bruker)

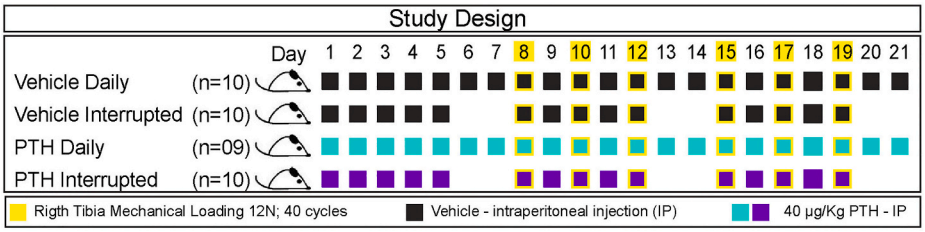

Fig. 1. Diagrammatic representation of the study design. This shows four mouse groups in each of which the right limb was loaded and left served as paired non-loaded control. Mice were either treated with: i) Vehicle daily ($n = 10$), Vehicle interrupted ($n = 10$), PTH daily (7/7; $n = 9$) or PTH with interruption (5/7; $n = 10$). PTH dosing was from day 1 to day 21 and, in each group, six mechanical loading episodes were applied to the right tibia (12 N; 40 cycles) during the last two weeks of treatment (yellow outlined boxes). Black denotes vehicle-treated, and turquoise and purple boxes denote 7/7 and 5/7 PTH-treated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

software in three steps: first, a region of interest (ROI) was manually drawn around the tibia to remove the femur, fibula, patella, fabellae and all bones of the foot. Second, the tibial image stack in the resulting ROI was loaded, and a new ROI was manually drawn to segment the trabecular bone from the proximal 6 % to 16 % of the total tibial length; this trabecular ROI included both primary and secondary spongiosa, but excluded all cortical bone. Finally, manual drawing was used to isolate the trabecular 'space'/ medullary area from the cortex along the tibia length, the ROI was subtracted, and the segmented cortical images were saved (Fig. 2). A lower threshold of 95 and an upper grey threshold of 255 were applied for cortical and trabecular bone images. Analyses of cortical (proximal 10–90 % of the tibia length) and trabecular bone (proximal 6–16 % of the tibia length) were performed using 2D image analysis in BatMan® (Bruker). Cortical bone parameters measured were cross-sectional area (CSA), ellipticity, and predicted resistance to torsion (J); trabecular bone parameters measured were bone area per total area (B·Ar/T.Ar- referred to as BV/TV when analysed in 3D), trabecular thickness (Tb.Th), and trabecular number (Tb.N).

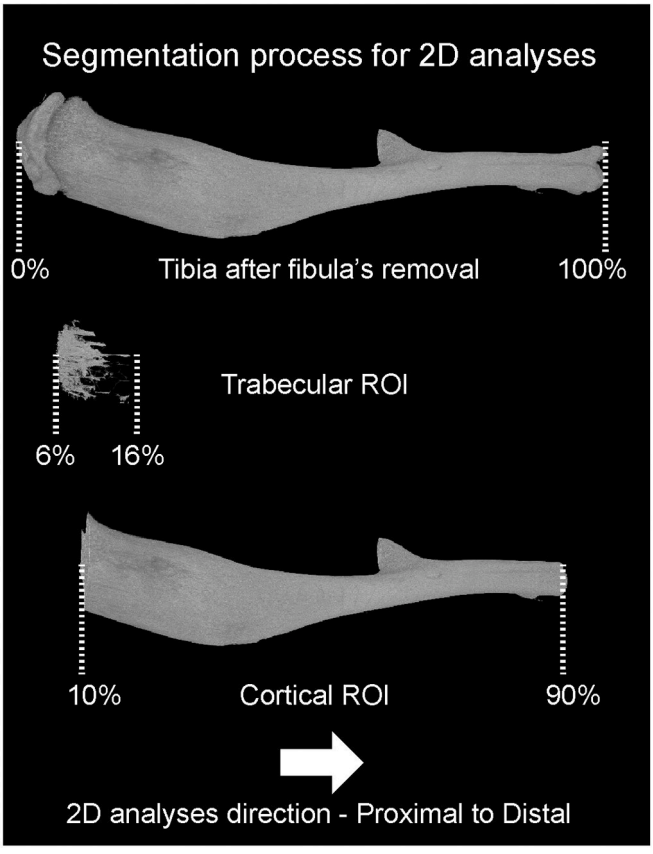

Fig. 2. Diagram demonstrating the segmentation process by which trabecular and cortical regions of interest are allocated. Each panel also contains the direction (right-to-left; proximal-to distal) of the 2D analyses and how percentage length is assigned.

2.8. Epiphyseal bone

We applied a semi-automated method [21] in Avizo® (Thermo Fisher Scientific, USA) software to segment and measure tibial epiphyseal cortical and trabecular bone and the marrow space volumes, as well as bone volume per total volume (BV/TV = Tb volume/Tb volume, plus marrow space volume).

2.9. Growth plate bridging analysis

Growth plate bone bridge analysis was performed on the tibia images using Avizo® (Thermo Fisher Scientific, USA) software. The central points of all bridges were identified and projected onto the tibial joint surface (see Fig. 10). The bridges number (N) and areal density (d), per 256 µm × 256 µm window, were calculated in each knee joint. 3D reconstructions of each joint, including the growth plate, were generated in Avizo, and each bridge, which crosses the growth plate to create a mineralised continuity between the epiphysis and metaphysis, is allocated a heat-map colour that represents the areal number density at each bridge location.

2.10. Statistical analysis

Whole bone 2D cross sectional analyses of the cortical and trabecular compartments (10–90 % and 6–16 % of tibial length, respectively) were performed in R software, version 4.0.2, using a linear mixed-effects model to assess the overall *fixed* effects of PTH treatment, mechanical loading and their interaction at each distinct percentile of the bone length. Individual mouse ID was included as a *random intercept* effect to account for repeated measures for the same mouse (loaded and non-loaded tibia). Shapiro-Wilk normality tests were performed to assess normality of residuals, and normality assumption of the data was assumed if most of the length (10–90 % of the tibial length) satisfied the null hypothesis of normality. Post-hoc comparisons between loaded and non-loaded effects within PTH treatment, or between PTH and vehicle effects within loading group were evaluated based on the estimated parameters from the mixed effects models. Data are presented as mean ± SEM and heatmaps that provide statistical significance ($p < 0.001$; $0.001 \leq p < 0.01$; $0.01 \leq p < 0.05$ and $p \geq 0.05$) at each location were also generated.

This study focuses on how PTH dosing regimens influence bones' loading-related responses. With view to pinpointing combinational treatment strategies that yield greater bone gains, we thus seek to identify circumstances where the two stimuli interact. Data depict regional changes induced by each treatment alone, and pinpoint where their combination engenders a change that is statistically different from their purely additive effects; termed 'interaction' (load:PTH interaction). We emphasise locations at which this interaction is 'synergistic', where the combined 'PTH plus loading' response is statistically *greater*

than a simple addition of each treatment applied alone. There are circumstances where load:PTH interaction (response to combined treatment) is statistically smaller than addition of each treatment alone. To better display differences in this interaction between the two PTH regimens, we have also plotted load-induced changes (loaded minus non-loaded effects on tibia) under basal (vehicle) and PTH treated conditions for each regimen.

Epiphyseal cortical, trabecular and marrow space volumes, BV/TV, bridge number and areal density were analysed using 2-way repeated measure ANOVA (loaded vs non-loaded repeated) followed by Sidak's multiple post-hoc comparison test. Normality of the residuals was assessed by visual inspection of QQ plot. All analyses were performed in GraphPad Prism® 9.3.0 software. Type I error rate was set at 0.05 for all analyses.

3. Results

3.1. Daily PTH enhances cortical bone mass and modifies shape along almost the entire tibia length and these effects are partly mitigated by a brief interruption in treatment

Examination of non-loaded left hind-limbs shows that daily PTH 7/7 elicits significant increases in cortical cross-sectional area (CSA, Fig. 3A) and predicted resistance to torsion (J, Fig. 4A) across vast regions (10–60 %) of the tibia length, with significant changes also extending to small regions distal to the tibiofibular junction (~66–83 %). Significant modifications in ellipticity were also concentrated in the proximal tibia (11–40 % of length), where daily PTH 7/7 produced shifts to a markedly round shaped cross-section (Fig. 5A). Interrupted PTH 5/7 treatment in non-loaded left hind-limbs also led to increases in CSA in similar cortical tibia regions as daily 7/7 treatment (Fig. 3B). In contrast, this interrupted PTH 5/7 treatment rendered the tibial mid-shaft region (50–60 %) and a region around 30 % of the tibia length, devoid of significant CSA increases (Fig. 3B). Furthermore, interrupted PTH 5/7 failed to exert significant modification in J in the cortex (Fig. 4B), which showed only minor changes at ~10 and ~40 % of the tibial length, with

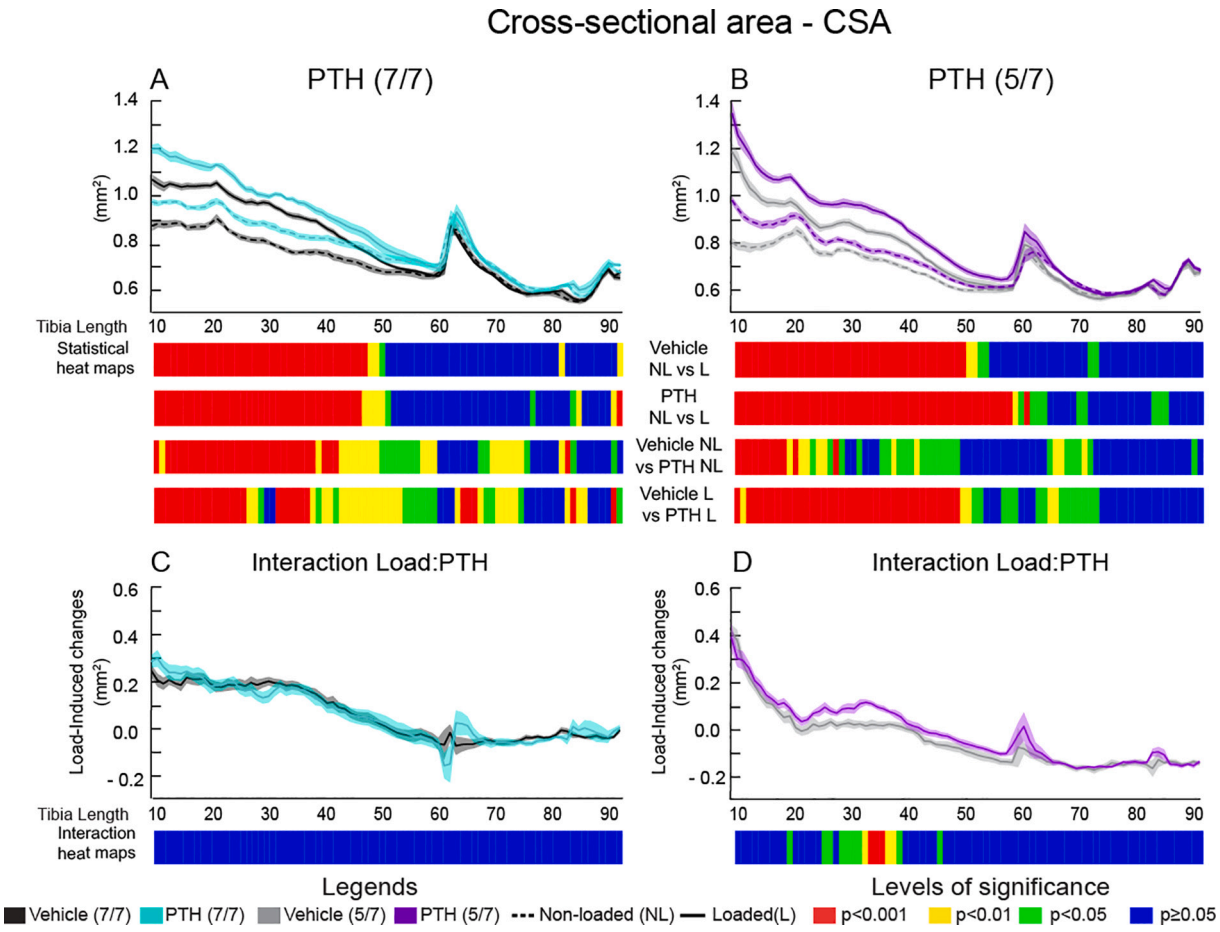

Fig. 3. Cross-sectional area. A and B: Cross-sectional area (mm^2) along the tibia (10–90 % of length) of left (non-loaded) and right (loaded) limbs from both vehicle- and PTH-treated mice, dosed using either daily 7/7 (A) or 5/7 (B) regimens. Mean ± standard error (with matching coloured shadowing) with heatmaps (below) showing statistical comparison at each percentile of length between: non-loaded (NL) and loaded (L) tibiae in vehicle- and PTH-treated mice (Vehicle NL vs L and PTH NL vs L, respectively), non-loaded tibiae from vehicle- and PTH-treated mice (Vehicle NL vs PTH NL), and loaded tibiae from vehicle- and PTH-treated mice (Vehicle L vs PTH L). C and D: Load-induced changes (loaded minus non-loaded) in vehicle- and PTH-treated mice using either the daily 7/7 dosing (C) or the interrupted 5/7 dosing regimen (D), with corresponding statistical heatmaps (below) showing statistical level of significance for 'Interaction Load:PTH' on a slice-by-slice basis along the tibia length. Group sizes were $n = 10$ for both Vehicle (7/7, black and 5/7, grey) and PTH (5/7, purple) treated mice, and $n = 9$ for PTH (7/7, turquoise) treated mice. Dashed lines show data for non-loaded tibiae and solid lines data for loaded tibiae. All comparisons exhibited normal distribution, and in heat maps red denotes $P < 0.001$; yellow, $P < 0.01$; green, $P < 0.05$; blue, $P \geq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

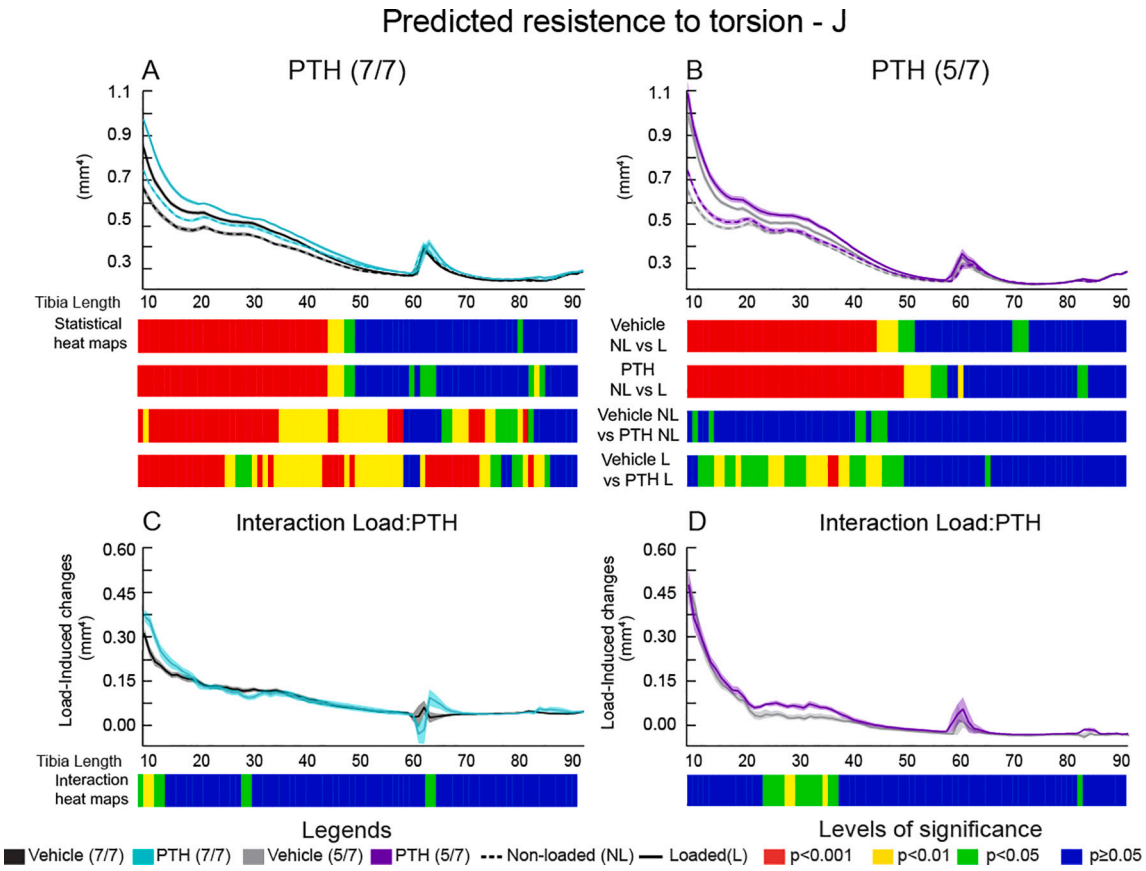

Fig. 4. Predicted resistance to torsion. A and B: Predicted resistance to torsion (mm^4) along the tibia (10–90 % of length) of left (non-loaded) and right (loaded) limbs from both vehicle- and PTH-treated mice, dosed using either daily 7/7 (A) or 5/7 (B) regimens. Mean ± standard error (with matching coloured shadowing) with heatmaps (below) showing statistical comparison at each percentile of length between: non-loaded (NL) and loaded (L) tibiae in vehicle- and PTH-treated mice (Vehicle NL vs L and PTH NL vs L, respectively), non-loaded tibiae from vehicle- and PTH-treated mice (Vehicle NL vs PTH NL), and loaded tibiae from vehicle- and PTH-treated mice (Vehicle L vs PTH L). C and D: Load-induced changes (loaded minus non-loaded) in vehicle- and PTH-treated mice using either the daily 7/7 dosing (C) or the interrupted 5/7 dosing regimen (D), with corresponding statistical heatmaps (below) showing statistical level of significance for 'Interaction Load:PTH' on a slice-by-slice basis along the tibia length. Group sizes were $n = 10$ for both Vehicle (7/7, black and 5/7, grey) and PTH (5/7, purple) treated mice, and $n = 9$ for PTH (7/7, turquoise) treated mice. Dashed lines show data for non-loaded tibiae and solid lines data for loaded tibiae. All comparisons exhibited normal distribution, and in heat maps red denotes $P < 0.001$; yellow, $P < 0.01$; green, $P < 0.05$; blue, $P \geq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ellipticity exhibiting a similar pattern of relatively diminished response with this interrupted compared to daily PTH 7/7 treatment (Fig. 5B).

3.2. *Mechanical loading enhances cortical mass and modifies tibia shape predominantly in regions proximal to the tibiofibular junction*

In accord with previous findings, application of six load episodes to right tibia was sufficient to provoke marked and significant increases in CSA and J between 10 and 50 % in both vehicle-treated groups of mice (Figs. 3A/B and 4A/B) [12,14]; near-identical responses to loading were observed in the two control groups (from the two dosing regimens). Load-induced changes in cortical ellipticity were slightly more concentrated to the proximal tibial regions (10–40 %), (Fig. 5A/B). It is noteworthy that the effects of mechanical load and of daily PTH 7/7 (see above) differ markedly in magnitude, yet both predominantly target the proximal half of the tibial cortex.

3.3. *Load: PTH interactions are regionally restricted and sensitive to PTH dosing regimen*

We next examined the extent to which any interaction was evident between the effects of loading and PTH in the cortical bone, under each of the two dosing regimens. Examination of changes in tibial CSA (Fig. 3A/B) and J (Fig. 4A/B) reveal that the combined effect of loading and PTH, administered either daily (7/7) or with interruption (5/7), promote more gains in CSA and J than either PTH or mechanical loading alone. In contrast, neither of these dosing regimens promote any greater shift in ellipticity (Figs. 5A/B) than mechanical load alone.

Comparisons between loaded, right tibiae from daily PTH (7/7) and matched vehicle-treated groups demonstrate that the coupled effect of daily PTH (7/7) with loading was evident in almost the whole tibia for CSA and J, extending distally well beyond the tibiofibular junction, leaving only minor regions (at ~60, 78 and 88 %) of the tibia length unmodified (Figs. 3A and 4A). Data showing load-induced changes (load minus non-loaded) with daily PTH (7/7) treatment show complete absence of any significant synergistic load:PTH interaction in CSA, anywhere along the tibia length (Fig. 3C); only sporadic load:PTH interaction at small, widely separated tibia regions were, likewise, evident in J (Fig. 4C). In contrast, comparison of load-induced changes between in vehicle-treated and interrupted PTH (5/7) groups revealed significant synergistic load:PTH interaction in the enhancement of CSA well as J (Figs. 3D and 4D). Neither PTH (7/7) nor (PTH 5/7) exposed synergistic interaction for ellipticity, as both graphs overlap (loaded limbs from PTH- and vehicle-treated mice; Fig. 5A/B); no clear evidence of synergy with either dosing regimen was seen in the load-induced changes in ellipticity (Fig. 5C/D). These data provide compelling evidence that uninterrupted daily PTH 7/7 significantly blunts the effects of load on tibial ellipticity; most markedly in most load-responsive

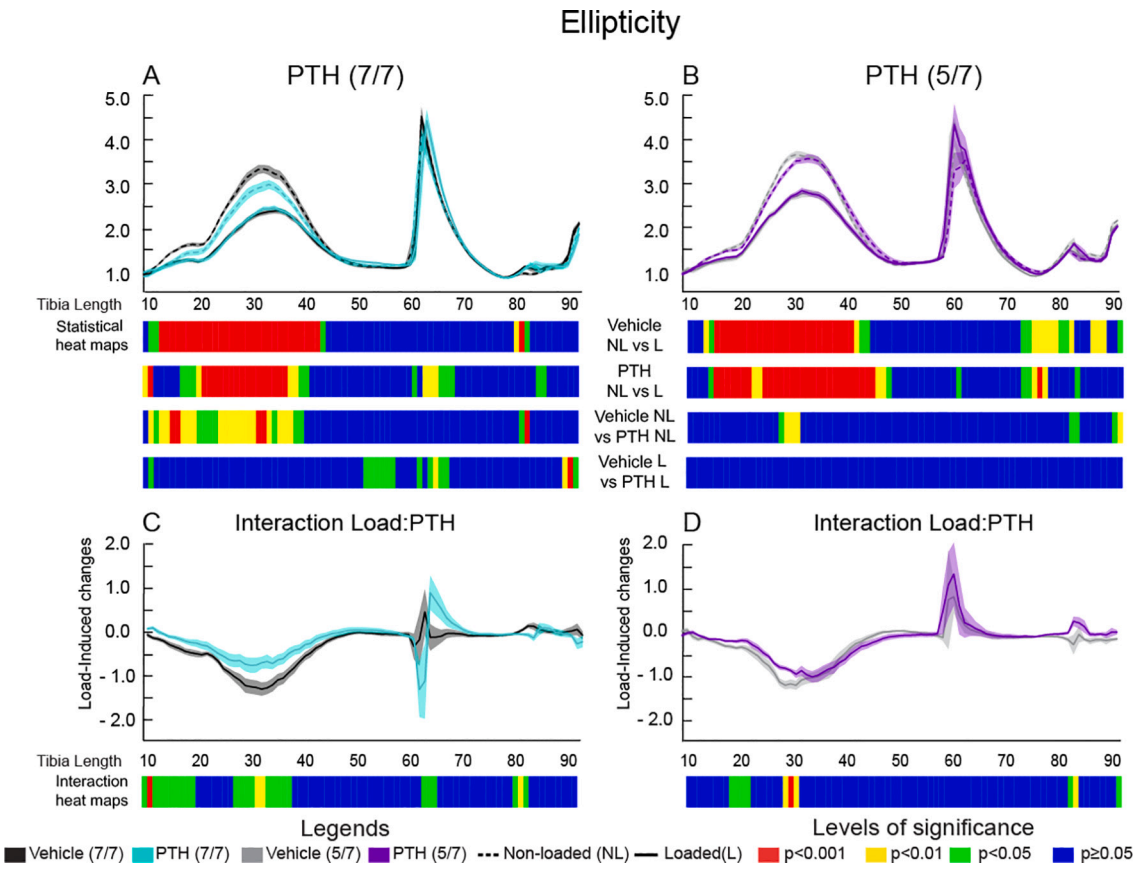

Fig. 5. Ellipticity. A and B: Ellipticity along the tibia (10–90 % of length) of left (non-loaded) and right (loaded) limbs from both vehicle- and PTH-treated mice, dosed using either daily 7/7 (A) or 5/7 (B) regimens. Mean ± standard error (with matching coloured shadowing) with heatmaps (below) showing statistical comparison at each percentile of length between: non-loaded (NL) and loaded (L) tibiae in vehicle- and PTH-treated mice (Vehicle NL vs L and PTH NL vs L, respectively), non-loaded tibiae from vehicle- and PTH-treated mice (Vehicle NL vs PTH NL), and loaded tibiae from vehicle- and PTH-treated mice (Vehicle L vs PTH L). C and D: Load-induced changes (loaded minus non-loaded) in vehicle- and PTH-treated mice using either the daily 7/7 dosing (C) or the interrupted 5/7 dosing regimen (D), with corresponding statistical heatmaps (below) showing statistical level of significance for 'Interaction Load:PTH' on a slice-by-slice basis along the tibia length. Group sizes were $n = 10$ for both Vehicle (7/7, black and 5/7, grey) and PTH (5/7, purple) treated mice, and $n = 9$ for PTH (7/7, turquoise) treated mice. Dashed lines show data for non-loaded tibiae and solid lines data for loaded tibiae. All comparisons exhibited normal distribution, and in heat maps red denotes $P < 0.001$; yellow, $P < 0.01$; green, $P < 0.05$; blue, $P \geq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proximal regions (Fig. 5A); similar PTH-related blunting of load-induced shifts in ellipticity are also seen with PTH 5/7 treatment.

There is also value in comparing the right loaded tibia to its left, contralateral non-loaded (internal) control in groups receiving either PTH 5/7 or PTH 7/7. Comparisons show that the PTH dosing regimen noticeably modified the tibial region in which mechanical load exerts its effect. Effects of load were shifted more distally in the PTH 5/7 group, most markedly in CSA for which changes extended to only <50 % of tibia length with daily PTH 7/7 (Supplementary Fig. 1A), but to ~65 % when load was combined with PTH 5/7 dosing (Supplementary Fig. 1B). Similar trends were evident for J (Fig. 11C/D -Supplementary) and ellipticity (Fig. 1E/F-Supplementary). These data indicate that the interaction between mechanical load and PTH influences a greater proportion of the tibia and is more marked when this interrupted, 'sub-optimal' PTH 5/7 dosing is used.

3.4. Daily PTH enhances trabecular architecture, but this is abolished by an interruption in dosing

Data from non-loaded, left tibiae show that daily PTH 7/7 elicited widespread and significant increases in trabecular bone area per total area (B·Ar/T.Ar,), thickness (Tb.Th,) and number (Tb.N, Figs. 6A, 7A and 8A, respectively) across almost the entire trabecular region examined (6–16 %). In contrast, an interruption in treatment for only two days in the PTH 5/7 group resulted in failure to elicit any major changes in trabecular architecture (Figs. 6B, 7B and 8B). These data indicate that the trabecular bone compartment is highly sensitive to even a brief interruption in PTH dosing.

3.5. Mechanical loading enhances trabecular architecture

Applied load alone elicited similar, yet non-identical changes in B. Ar/T.Ar, Tb.Th and Tb.N in both vehicle-treated mouse groups (Figs. 6A/B, 7A/B and 8A/B). These loading-induced increases were distributed across most of the trabecular region examined (6–14 %). The only parameter that did not follow this pattern was Tb.N, which showed minor shifts in response to applied load (8–14 %, Fig. 8B) in the experimental group receiving the PTH 5/7 regimen.

3.6. Daily and interrupted PTH interact with mechanical load to engender both region-specific gains and losses in trabecular bone mass

Increases in B.Ar/T.Ar, Tb.Th and Tb.N were consistently more marked and widespread with daily PTH (7/7) and almost completely absent with interrupted PTH (5/7) (Figs. 6A/B, 7A/B and 8A/B, see Supplementary Figs. 2 and 6–8). Application of loading alone was sufficient to provoke marked near-identical increases in B.Ar/T.Ar, Tb.Th and Tb.N mostly concentrated in the more proximal trabecular region in

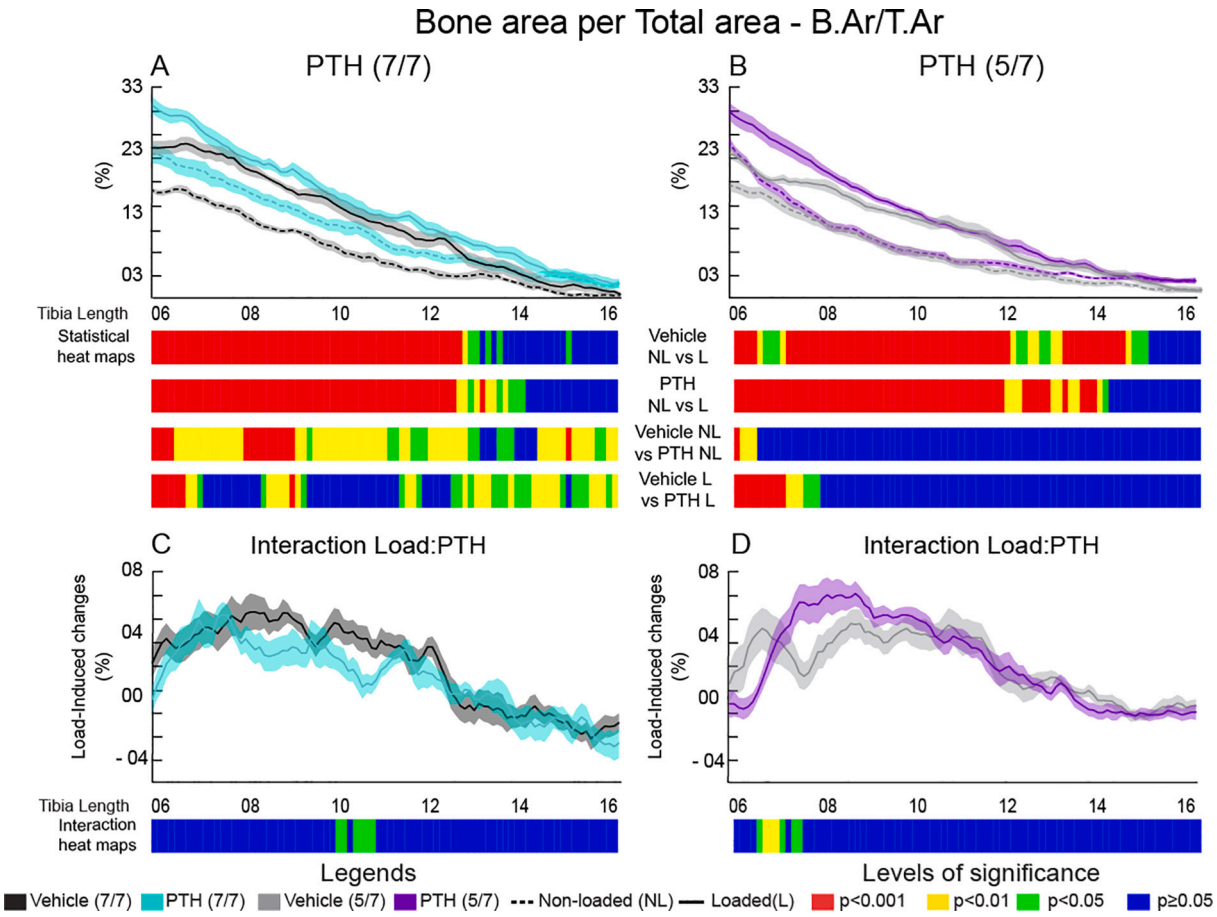

Fig. 6. Bone area per total area. A and B: Bone area per total area (%) along the tibia (06–16 % of length) of left (non-loaded) and right (loaded) limbs from both vehicle- and PTH-treated mice, dosed using either daily 7/7 (A) or 5/7 (B) regimens. Mean ± standard error (with matching coloured shadowing) with heatmaps (below) showing statistical comparison at each percentile of length between: non-loaded (NL) and loaded (L) tibiae in vehicle- and PTH-treated mice (Vehicle NL vs L and PTH NL vs L, respectively), non-loaded tibiae from vehicle- and PTH-treated mice (Vehicle NL vs PTH NL), and loaded tibiae from vehicle- and PTH-treated mice (Vehicle L vs PTH L). C and D: Load-induced changes (loaded minus non-loaded) in vehicle- and PTH-treated mice using either the daily 7/7 dosing (C) or the interrupted 5/7 dosing regimen (D), with corresponding statistical heatmaps (below) showing statistical level of significance for 'Interaction Load:PTH' on a slice-by-slice basis along the tibia length. Group sizes were $n = 10$ for both Vehicle (7/7, black and 5/7, grey) and PTH (5/7, purple) treated mice, and $n = 9$ for PTH (7/7, turquoise) treated mice. Dashed lines show data for non-loaded tibiae and solid lines data for loaded tibiae. All comparisons exhibited normal distribution, and in heat maps red denotes $P < 0.001$; yellow, $P < 0.01$; green, $P < 0.05$; blue, $P \geq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

both vehicle-treated groups of mice (Figs. 6A/B, 7A/B and 8A/B). Loading failed to exert any more marked interaction with PTH in trabecular bone when applied in 5/7 than 7/7 regimen; only small regions exhibited interaction. A specific suppressive load:PTH interaction pattern was evident in the most proximal trabecular region (<7 %) where, for example, Tb.Th was lower in the group with combined load and either of the two PTH dosing regimens, than with load alone. Load:PTH interactions in B.Ar/T.Ar, Tb.Th and Tb.N were not conserved across the trabecula ROI (Figs. 6-8, C and D). Comparisons of loaded and non-loaded tibiae demonstrated that 5/7 and 7/7 PTH produced similar regional modifications in B.Ar/T.Ar, Tb.Th and Tb.N across most of the trabecular region (6–16 %; Supplementary Fig. 2). Thus, load:PTH interaction in trabecular bone does not resemble cortical responses as they were neither greater nor more widespread with interrupted, 'sub-optimal' PTH than daily dosing.

3.7. PTH 5/7 but not mechanical loading modifies epiphyseal bone

To assess if load:PTH interaction is conserved in diverse bone compartments, we also examined tibial epiphyses in mouse groups receiving PTH 5/7 treatment (vs vehicle control). Semi-automated analysis of the entire proximal tibia epiphysis showed that PTH 5/7 produced significant increases in epiphyseal cortical volume and trabecular BV/TV, as well as a decreases in marrow space volume in both loaded and non-loaded tibiae (Fig. 9, Supplementary Table 1).The epiphyseal bone was however apparently completely insensitive to applied loading alone. In the other hand, the amalgamation of PTH and mechanical loading decreased the cortical volume at the loaded epiphyses, such modification seems to originate from a negative interaction between load:PTH, as loading alone failed to induce any change (Fig. 9, Supplementary Table 1).

3.8. Mechanical loading, but not PTH, reduces the number and areal density of bridges

To explore whether alternative sites of skeletal mineralisation exhibit modified response to loading and PTH, we also examined bony 'bridge' structures in the cartilaginous epiphyseal growth plate region in loaded, and non-loaded growth plates in PTH 5/7 treated groups [22]. Counting of the number and mapping of the areal density of the mineralised bony bridges showed that PTH 5/7 treatment did not significantly modify either their total number or areal density. In contrast, we found that loading significantly diminished both GP bridge number and areal density, but did not generate any load:PTH interaction in GP

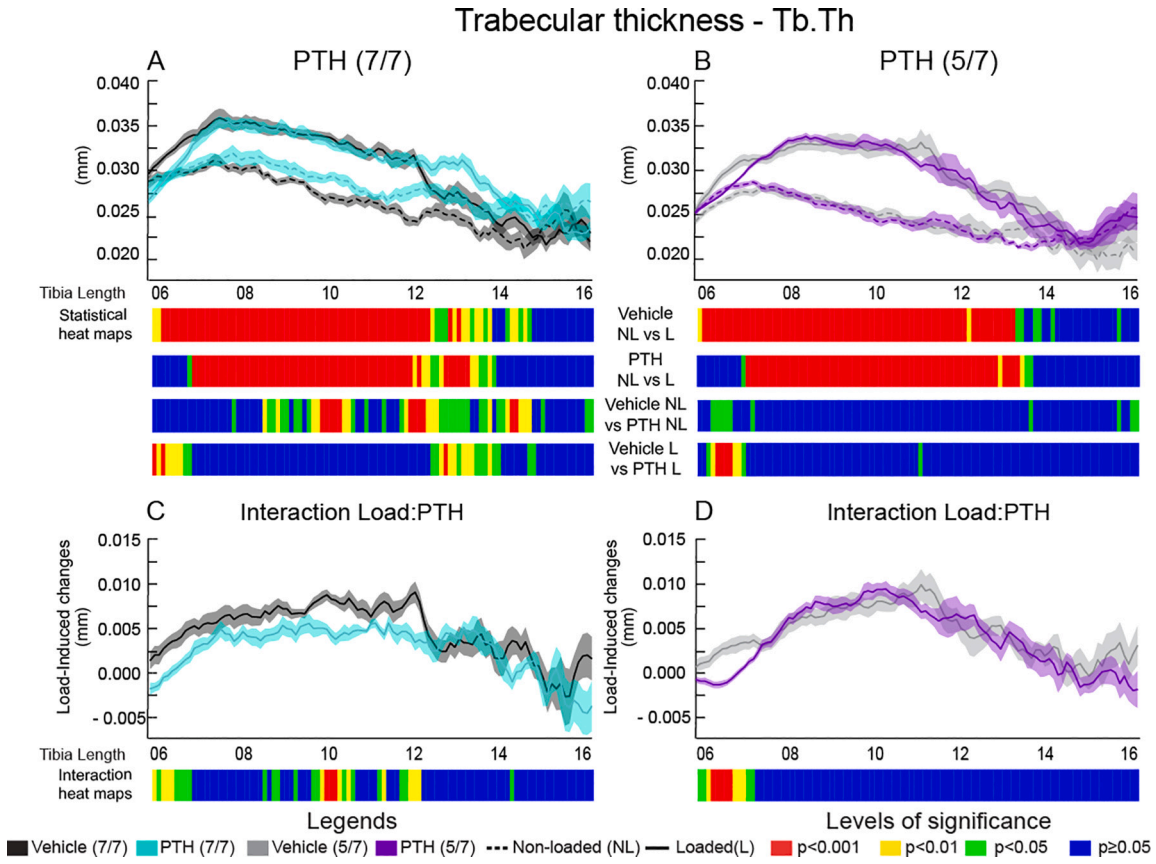

Fig. 7. Trabecular thickness. A and B: Trabecular thickness (mm) along the tibia (06–16 % of length) of left (non-loaded) and right (loaded) limbs from both vehicle- and PTH-treated mice, dosed using either daily 7/7 (A) or 5/7 (B) regimens. Mean ± standard error (with matching coloured shadowing) with heatmaps (below) showing statistical comparison at each percentile of length between: non-loaded (NL) and loaded (L) tibiae in vehicle- and PTH-treated mice (Vehicle NL vs L and PTH NL vs L, respectively), non-loaded tibiae from vehicle- and PTH-treated mice (Vehicle NL vs PTH NL), and loaded tibiae from vehicle- and PTH-treated mice (Vehicle L vs PTH L). C and D: Load-induced changes (loaded minus non-loaded) in vehicle- and PTH-treated mice using either the daily 7/7 dosing (C) or the interrupted 5/7 dosing regimen (D), with corresponding statistical heatmaps (below) showing statistical level of significance for 'Interaction Load:PTH' on a slice-by-slice basis along the tibia length. Group sizes were $n = 10$ for both Vehicle (7/7, black and 5/7, grey) and PTH (5/7, purple) treated mice, and $n = 9$ for PTH (7/7, turquoise) treated mice. Dashed lines show data for non-loaded tibiae and solid lines data for loaded tibiae. All comparisons exhibited normal distribution, and in heat maps red denotes $P < 0.001$; yellow, $P < 0.01$; green, $P < 0.05$; blue, $P \geq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bridge regulation (Fig. 10, Supplementary Table 2).

4. Discussion

Our studies show that the intermittency of PTH dosing regimens can modify interaction with loading for greater anabolic impact. We find that daily PTH (7/7) retains an interaction with load in the changes in tibia shape (ellipticity and J), but obscures any interaction with load in the regulation of cortical mass. Thus, insertion of a simple 2 day-long interruption in PTH (5/7) dosing discloses an interaction with load that serves to synergistically enhance cortical mass across extensive tibia regions. PTH-induced trabecular mass increases are also restricted by interrupted PTH (5/7) treatment, but interaction with loading occurs in both dosing regimens. We also find that this load:PTH interaction is regionally-restricted across these cortical and metaphyseal sites, and less evident in the epiphysis.

Transient interruptions in PTH delivery are critical. Continuous PTH infusion is catabolic and promotes bone resorption via serum amyloid A, but intermittent dosing triggers an anabolic *window* [7,23,24]. This implies pivotal roles for multiple, transient rises (and/or falls) in PTH levels generated by daily administration. It is notable that this inference is justified only with PTH administration in the context of exclusively habitual loads and that synergism in cortical responses are greater when PTH is delivered with 2 day-long interruptions. Our data reinforce strong links between the mechanism of anabolic action of load and intermittent PTH [4,17,19] and may explain how both additive as well as synergistic load:PTH interactions have been observed previously.

Early studies exploring load:PTH interaction in cortical bone regulation were conducted in rats. Using axial tibia loading and 4-point ulna bending in intact 19 month-old rats, Li et al. [25] found that short-term co-administered PTH with a single load episode elicited synergistic increases in endocortical and periosteal bone accrual at sites of maximum strain in the tibia and ulna cortex, respectively [25]. Consistent with this, Sugiyama et al. [20] found that 6 episodes (2 weeks) of low magnitude tibia loading (2.5 N) in intact 13 week-old mice, which was insufficient for an anabolic response, synergised in the distal tibia cortex with 4 week-long, high dose uninterrupted daily PTH, and that higher anabolic loading (12 N) extended this synergy proximally [20]. Closely aligned studies using lower PTH doses in aged mice, however, found that such load:PTH co-treatment exerted only additive (not synergistic) effects in the periosteal cortices [5]. Likewise, mice subjected to six 12 N episodes of tibia loading, 5 weeks post-ovariectomy, showed: i) no load:PTH synergy and purely additive effects on periosteal apposition in only the proximal tibia [18], ii) additive increases in tibia stiffness and strength from finite element analysis [19], and iii) only additive benefit in mid-diaphyseal tibial cortices where strains are high [4]. On balance, these data support a synergistic load:PTH interaction in the cortical bone of young, intact mice which decays to solely additive benefit in aged or

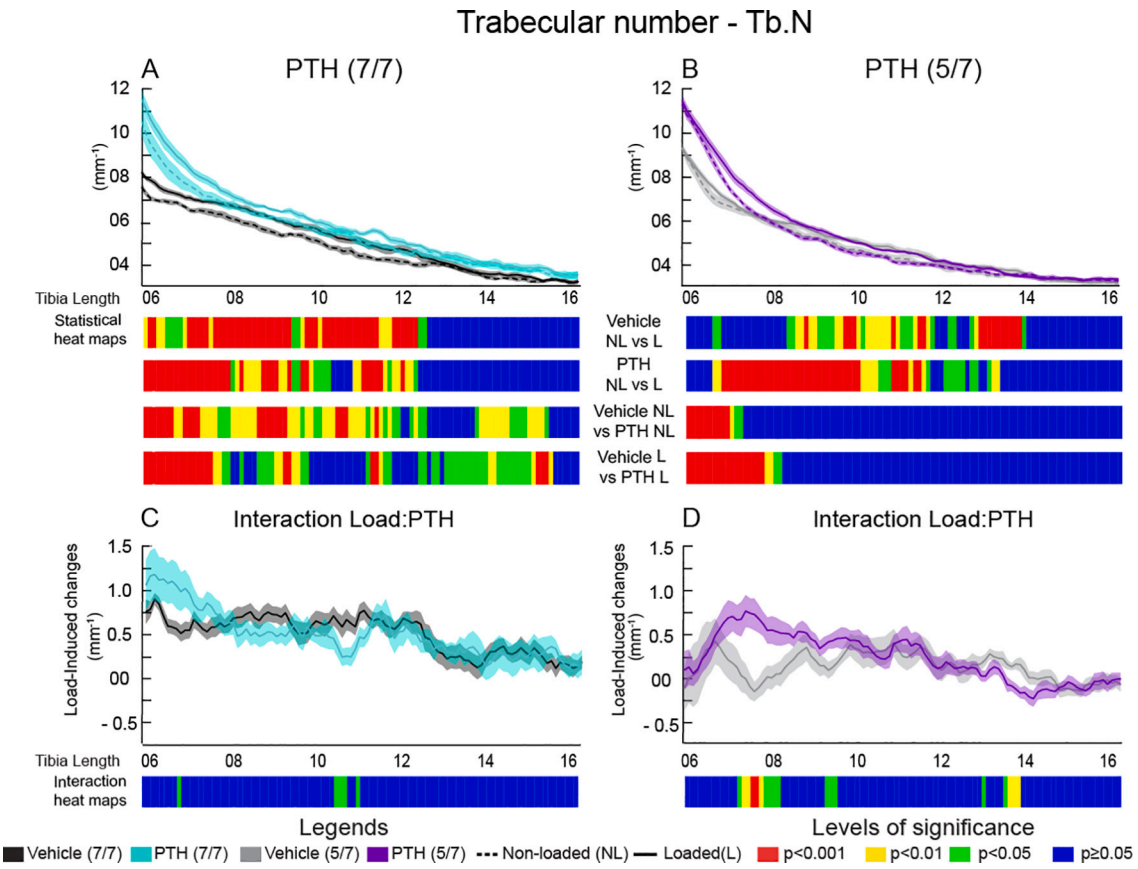

Fig. 8. Trabecular number. A and B: Trabecular number (mm^{-1}) along the tibia (06–16 % of length) of left (non-loaded) and right (loaded) limbs from both vehicle- and PTH-treated mice, dosed using either daily 7/7 (A) or 5/7 (B) regimens. Mean ± standard error (with matching coloured shadowing) with heatmaps (below) showing statistical comparison at each percentile of length between: non-loaded (NL) and loaded (L) tibiae in vehicle- and PTH-treated mice (Vehicle NL vs L and PTH NL vs L, respectively), non-loaded tibiae from vehicle- and PTH-treated mice (Vehicle NL vs PTH NL), and loaded tibiae from vehicle- and PTH-treated mice (Vehicle L vs PTH L). C and D: Load-induced changes (loaded minus non-loaded) in vehicle- and PTH-treated mice using either the daily 7/7 dosing (C) or the interrupted 5/7 dosing regimen (D), with corresponding statistical heatmaps (below) showing statistical level of significance for 'Interaction Load:PTH' on a slice-by-slice basis along the tibia length. Group sizes were n = 10 for both Vehicle (7/7, black and 5/7, grey) and PTH (5/7, purple) treated mice, and n = 9 for PTH (7/7, turquoise) treated mice. Dashed lines show data for non-loaded tibiae and solid lines data for loaded tibiae. All comparisons exhibited normal distribution, and in heat maps red denotes $P < 0.001$; yellow, $P < 0.01$; green, $P < 0.05$; blue, $P \geq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ovariectomised mice. This may inform clinical advantage of combined PTH pharmacotherapy and bone loading in osteoporotic post-menopausal and aged patients.

It is intriguing, therefore, that employment of time-lapsed micro-CT to track (re)modelling in intact, 16 week-old female C57Bl/6 mice with interrupted PTH dosing (5/7) and longer-term loading (5 day/week, 5 weeks) at lower (9 N) loads, showed evidence of local synergy in the anabolic (periosteal) and catabolic modelling (endosteal) of cortices and remodeling of trabecular bone [17]. This makes it conceivable that bespoke interruptions in PTH dosing and timed exposure to exercise-induced mechanical loading may capture this synergism. This possibility is indeed supported by our data showing that PTH delivered with 2 day-long interruptions in dosing reveals synergism with applied loads that would otherwise remain cryptic. This benefit is particularly pertinent in the context of the findings of McAteer et al [26] which showed that 6 weeks of daily uninterrupted PTH exerted much more widespread changes in tibial mass and architecture than 6 loading episodes (over 2 weeks), and that although smaller gains were accrued in loaded bones, these converted to similar benefits in load-bearing strength [26]. It is pertinent to acknowledge that in our studies, the tibial response to loading in the two vehicle-treated groups, which serve as internal controls, diverges somewhat in the most proximal region in mice treated under the two dosing regimens. This serves to highlight the importance of including matched 'control' loaded groups in studies where interactions between load and systemically acting osteotrophic factors are being examined.

In addition to comparing daily and interrupted PTH treatment, our data suggest that there is benefit from regional evaluation and their interaction with load in the tibia cortical bone. Our data align with those of McAteer et al. [26] as we find that PTH enhances cortical mass along nearly the entire tibia, while the effects of mechanical loading are largely restricted to regions proximal to the tibiofibular junction and, compellingly, that their synergistic interaction is mostly restricted to more proximal regions for increases in cortical bone area (with interrupted PTH) and shape changes (with either dosing regime). These data highlight the possibility that the study of local changes in bone cell behaviour at these specific locations may provide a mechanistic understanding of the pathways involved in the osteogenic effects of PTH, of loading, and of the overlapping cellular signalling cascades which may underpin their additive or synergistic interaction.

Studies have found that cortical and trabecular bone can also behave differently in response to anabolic pharmacological agents and to changes in the mechanical milieu; this has been shown both in sciatic neurectomy-induced disuse and responses to PTH administration and loading [5,27]. Meakin et al. [5] showed that 25 or 50 μg/kg daily PTH is capable of modifying cortical, but not trabecular bone in aged female mice. In our work using younger female mice, daily administration of 40 μg/kg PTH significantly modified trabecular bone, suggesting that this

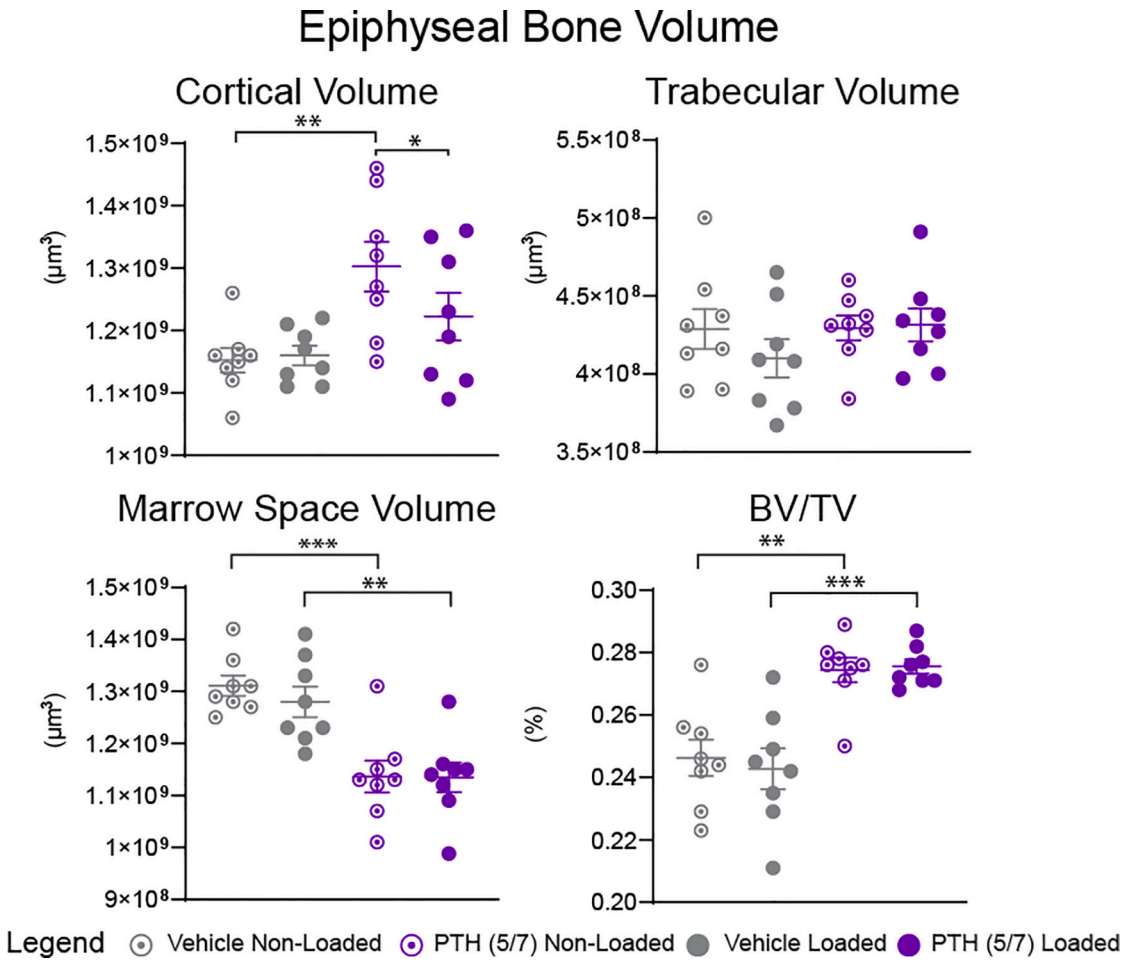

Fig. 9. Epiphyseal cortical, trabecular and marrow space volume, and bone volume per total volume in left and right tibiae of vehicle- and PTH- (5/7) treated mice in response to 3 weeks of dosing and 6 episodes of right-limb mechanical loading. Group sizes were $n = 8$ for Vehicle and PTH treated mice. All comparisons exhibited normal distribution and statistical analyses were made through 2-way repeated measure ANOVA (loaded vs non-loaded repeated) followed by Sidak's multiple post-hoc comparison test for epiphyseal cortical, trabecular and marrow space volume, and bone volume per total volume within the vehicle- (grey) and PTH-treated (purple) groups, and represented as different levels of significance as * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$. Open circles = non-loaded; closed circles = loaded. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

difference in trabecular sensitivity could be attributed to bone metabolism changes that occur with animal ageing [28]. On the other hand, we find that the 2 day-long interruption in PTH dosing regime is sufficient to result in the apparent loss of these PTH-related bone gains in the trabecular compartment. These data are consistent with a previous work that reported a 3D analysis from the trabecular compartment after treatment with PTH (5/7) and loading and highlighted that changes in this compartment were higher when loading was applied alone [4]. Our data extend these to show that mechanoadaptation of the trabecular bone compartment, as well as its sensitivity to interaction with PTH, differs regionally along the proximodistal metaphyseal axis, with evidence of negative interaction near, and positive interaction at sites more distant from the growth plate. This aligns with previous findings and may explain how both positive and negative trabecular load:PTH interactions have been observed in earlier studies [4,5,17].

Thus, Robinson et al. [17] described a suppressive 'tempering' effect of interrupted (5/7) PTH in mechanically loaded bones. Our 2D analyses of the trabecular compartment across the 6–16 % length of the tibia provide support for this negative PTH effect on loaded trabecular bone, yet emphasise its extreme restriction to the 6–7 % region of this compartment. This comprises high concentrations of primary spongiosa and whether it highlights differential sensitivity to PTH in primary and secondary spongiosal regions remains to be fully explored; as primary spongiosal trabeculae are thinner, this may be more vulnerable to resorption than thick trabeculae during PTH/mechanical load treatments [29].

As we found synergistic interaction between interrupted PTH (5/7) and loading in the control of cortical bone mass, we opted to evaluate only tibial epiphyses from this mouse group in order to explore if this interaction was specific to particular bone compartments. Previous work using an interrupted dosing regimen (5/7) had shown that PTH exerted dose-dependent modifications to epiphyseal architecture, with 40 µg/kg (but not 10 µg/kg) resulting in enhanced trabecular BMD, BV/TV and SMI [11]. In alignment with these studies, our data showed that interrupted PTH (5/7) dosing increased trabecular BV/TV but also increased epiphyseal cortical bone volume and correspondingly decreased marrow space volumes in the epiphyseal compartment. Given the apparent absence of such increases in metaphyseal trabecular regions in mice receiving the interrupted PTH (5/7) dosing, these data point to differential PTH sensitivities across these distinct bone compartments.

These bone type-specific responses are further supported by the lack of any load-induced modification in any of the epiphyseal bone parameters investigated, when applied alone and compared to non-loaded control limbs. It has been observed, however, that mechanical load is capable of inducing marked changes in the epiphyseal bone, but that such modifications rely on the frequency/duration of these applied loads; 5 weeks of 3 loading episodes each week were sufficient to engender increased epiphyseal cortical thickening and trabecular BV/

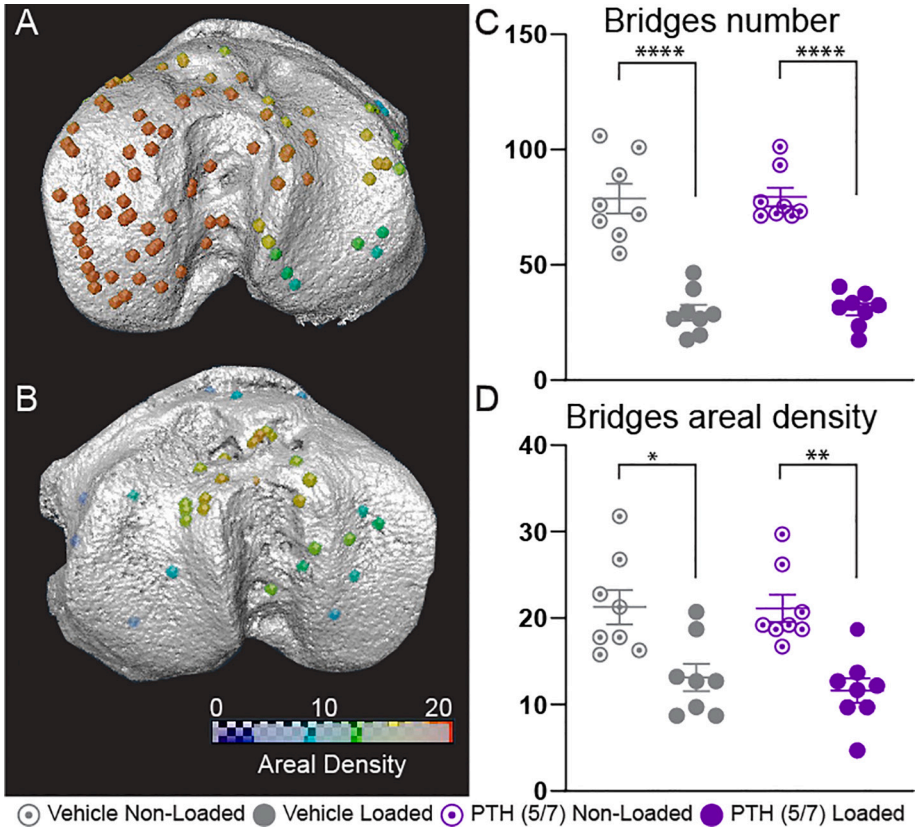

Fig. 10. Number of bridges and bridges areal density in left and right tibia from vehicle (grey) and PTH (5/7) treated mice (purple) in response to 3 weeks of dosing and 6 episodes of loading. A: PTH (5/7) Non-loaded left epiphyseal bone. B: PTH (5/7) loaded right epiphyseal bone. C: Number of bridges. D: Bridges areal density. Group sizes were $n = 08$ for Vehicle and PTH treated mice. All data exhibited normal distribution. Statistical analyses were made through 2-way repeated measure ANOVA (loaded vs non-loaded repeated) followed by Sidak's multiple post-hoc comparison test within the Vehicle and PTH, and represented as different levels of significance as * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, and **** $p < 0.0001$. Open circles = non-loaded; closed circles = loaded. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TV but a single episode or 6 episodes in two weeks did not [30]. It is likely that the somewhat brief loading exposure over two weeks in our work was insufficient to elicit changes in the epiphyseal bone. These data confirm differential PTH sensitivities across these distinct bone compartments and suggest that mechanical loading reverses the anabolic effect of PTH in the epiphyseal cortical volume when both treatments are applied together. Further analyses to better describe the epiphyseal bone changes induced by PTH and by loading, as well as their combined effects are clearly required.

With view to exploring load:PTH relationships in alternative skeletal mineralisation sites, we also measured cartilaginous epiphyseal growth plate 'bridge' structures in loaded and non-loaded limbs in mice receiving PTH (5/7) dosing. Bridge formation in mice is known to be related to age and to linear growth, making questions about regulation in their number and areal density by PTH and mechanical loading compelling. We used a recent image analyses technique developed in Aviso® software [22] that failed to reveal any evidence in intact female mice for regulation in growth plate bridge number or areal density in response to 3 weeks of PTH treatment. Using identical methods, bridge number and areal density have been reported to be greater on the medial aspect of the tibia than on the lateral, and that neither was modified by the induction of osteoarthritis in 16 week-old male mice via either surgical destabilisation of medial meniscus or non-invasive applied joint loading. Our data contrasts with these findings by revealing that 6 loading episodes were sufficient to markedly reduce bridge number in younger 11–13 week-old female C57BL6 mice. These inconsistent findings may be attributable to differences in age and/or sex.

It must be acknowledged that the interrupted (5/7) treatment regimen exposes mice to six fewer PTH injections than daily PTH (7/7). This reduced number of PTH doses could contribute to the differences that we have found between groups and, any extension in time only, so as to match the total number of doses, would have necessitated divergent total treatment duration, making comparison impossible. Another possible solution to more closely match our groups, would have been to increase concentration of each dose in the PTH 5/7 group from 40 μg/kg to 56 μg/kg, so that the cumulative dose was equivalent in both groups (this equivalence could have likewise been achieved by decreasing PTH concentration in the daily dosed group). This kind of adjustment would, however, had restricted evaluation against prior findings and would have compromised comparison between the daily and interrupted treatment groups, as the response to PTH is dependent upon the concentration of each dose [5,11,20]. Our choice to start PTH treatment one week prior to a fortnight of applied loading, was made in order to best replicate the earlier studies in which somewhat anomalous outcomes had been reported [4,5,19,20]; this would allow testing of our hypothesis that variation in the reported anabolic load:PTH interaction was due to the influence of differing PTH dosing regimens. It remains possible that the duration of the PTH dosing period, imposed prior to the start of loading, may also influence this interaction, as Meakin et al [5] performed loading in the last two weeks during a 2 or 6 week-long period of daily (7/7) treatment and reported robust effects at the 6 week timepoint in aged female mice.

Our study has limitations. In addition to using two dosing regimens in which the cumulative dose does not match, our study design also limits the direct comparison between PTH (7/7) and PTH (5/7) treatment regimens, as each was twinned to a group of vehicle-treated mice in which experimental dosing procedures was to be matched. Another limitation includes the use of contralateral tibiae, rather than tibiae from a separate group of non-loaded mice, as controls. It has been reported that mechanical loading exerts systemic effects on bone formation that may complicate the interpretation of our data. Choosing of contralateral limbs as controls was based partly on *reduction* in animal numbers used, partly on widespread studies of murine bone loading in which left tibiae have been deemed fitting internal controls, and partly upon studies refuting these reported systemic effects [4,5,12,14,31]. Another limitation is our use of a cross-sectional rather than longitudinal study design. Longitudinal in vivo scanning can provide valuable information, as it permits exploration of the trajectory of bone changes over the

experimental time-course, which can help to define potential mechanisms of action [17]. In vivo scanners do, however, tend to function at lower-resolution, with movement artefacts which impact on the capacity to resolve small bony details. Our cross-sectional studies with high-resolution images facilitates scope to identify smaller magnitude changes to bone architecture [17,18]. This may indeed underpin the capacity for our study to distinguish between the effects of uninterrupted and interrupted PTH regimens. We opted to use young adult female mice as they are known to be more responsive to loading than aged females; further studies could explore these interactions in older animals and in males for greater clinical relevance [1,5,13], but those which have done so have not found evidence of load:PTH synergy [5]. Finally, mice unlike humans, do not provide a ready tool for investigating intracortical remodeling, which may have great relevance to clinical translation [3,32]. PTH treatments in humans are FDA-approved for up to two years, with recent primate research supporting the hypothesis that there will not be detrimental effects of treatment withdrawal on bone gains [9]. Further studies are required to explore if combined PTH dosing with exercise regimens can lead to new, personalised therapies for osteoporotic patients. It would be fitting if skeletal sites that are most prone to osteoporotic fractures, namely the hip, spine and forearm [2], could be targeted by novel therapeutic approaches. Our findings showing that distinctive compartments within a single bone respond differently to combined PTH treatment with mechanical loading, suggests that such targeting may be possible. Our data also suggest that sedentary patients are likely to gain sub-optimal benefit from any interruption in their daily PTH intake.

5. Conclusion

Our research demonstrates that the interaction between PTH treatment and mechanical loading is dose-regimen, parameter- and region-specific in the mouse tibia. The interrupted PTH (5/7) regime had a synergic interaction with loading on bone mass gains, while both dosing regimens interacted with loading to spatially alter regions of cortical and trabecular bone architecture. As the tibia is a modular organ, both PTH and mechanical loading treatments were capable of modifying both the cortical and trabecular compartments, separately. In the tibial epiphysis, only PTH treatments enhanced bone volume, while mechanical loading decreased both the cortical volume only, in the presence of PTH, and the number of growth plate bridges. Our data provide new insights into the future potential for anabolic treatments of osteoporosis which could target specific bone regions, personalised for each patient in accordance with both their medical requirements and lifestyle.

CRediT authorship contribution statement

Samuel Monzem: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - Original Draft.

Dionysia Valkani: Conceptualization, Methodology, Investigation, Formal analysis.

Lucinda Anastasia Elizabeth Evans: Conceptualization, Methodology, Investigation.

Yu-Mei Chang: Formal analysis, Visualization.

Andrew Anthony Pitsillides: Conceptualization, Methodology, Resources, Supervision, Funding acquisition, Writing - Review & Editing.

All authors reviewed and approved the final version of the manuscript.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2023.116720.

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