

Maternal Bone Adaptation to Mechanical Loading during Pregnancy, Lactation, and Post-Weaning Recovery

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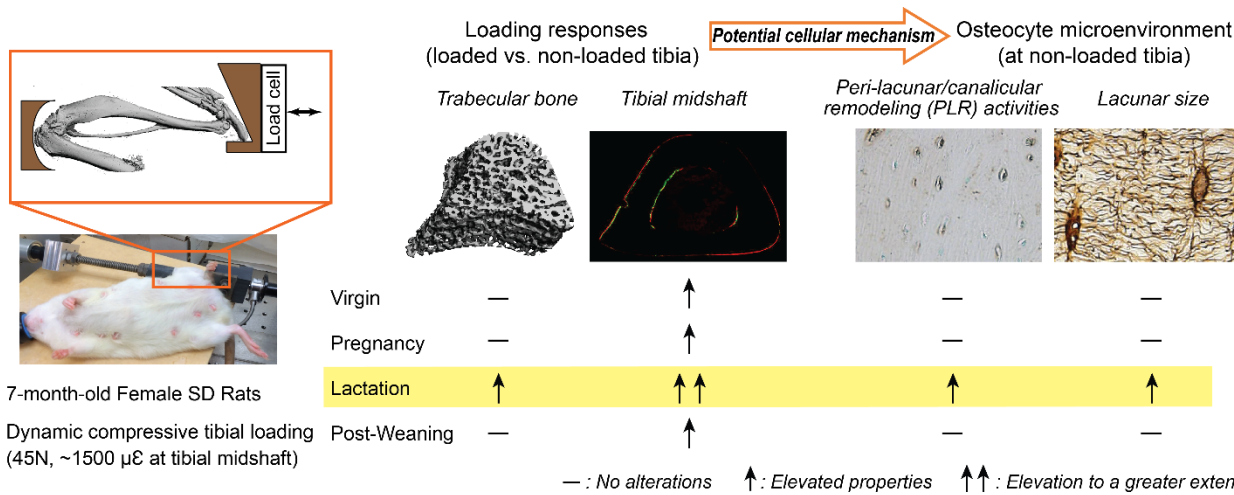
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Abstract:

The maternal skeleton undergoes dramatic bone loss during pregnancy and lactation, and substantial bone recovery post-weaning. The structural adaptations of maternal bone during reproduction and lactation exert a better protection of the mechanical integrity at the critical load-bearing sites, suggesting the importance of physiological load-bearing in regulating reproduction-induced skeletal alterations. Although it is suggested that physical exercise during pregnancy and breastfeeding improves women's physical and psychological well-being, its effects on maternal bone health remain unclear. Therefore, the objective of this study was to investigate the maternal bone adaptations to external mechanical loading during pregnancy, lactation, and post-weaning recovery. By utilizing an *in vivo* dynamic tibial loading protocol in a rat model, we demonstrated an improved maternal cortical bone structure in response to dynamic loading at tibial midshaft, regardless of reproductive status. Notably, despite the minimal loading responses detected in the trabecular bone in virgins, the rat bone during lactation experienced enhanced mechano-responsiveness in both trabecular and cortical bone compartments when compared to rats at other reproductive stages or age-matched virgins. Furthermore, our study showed that the lactation-induced elevation in osteocyte peri-lacunar/canalicular remodeling (PLR) activities led to enlarged osteocyte lacunae. This may result in alterations in interstitial fluid flow-mediated mechanical stimulation on osteocytes and an elevation in solute transport through the lacunar-canalicular system (LCS) during high-frequency dynamic loading, thus enhancing mechano-responsiveness of maternal bone during lactation. Taken together, findings from this study provide important insights into the relationship between reproduction and lactation-induced skeletal changes and external mechanical loading, emphasizing the importance of weight-bearing exercise on maternal bone health during reproduction and postpartum.

Keywords: Maternal bone adaptation; Bone mechano-responsiveness; Reproduction; Lactation; Peri-lacunar/canalicular remodeling (PLR); Osteocyte mechanosensing

Graphic Abstract:



1. Introduction

During reproduction and lactation, the maternal skeleton serves as an important reservoir for minerals, notably calcium and phosphorus, which can be accessed to allow the body to support fetal/infant growth and maintain homeostasis [1]. In women, the resulting skeletal resorption can lead to 1-4% decline in areal bone mineral density (aBMD) during pregnancy [2-6], and 4-8% reduction in BMD during lactation [6-11]. More strikingly, rodent studies have shown that lactation can result in 20-80% reduction in bone mass due to higher calcium requirement by the larger litter size in mice and rats [1, 12, 13]. Following weaning, the maternal skeleton undergoes a remarkable anabolic phase, leading to substantial recovery of bone mass and bone microstructure [1, 14-17]. Intriguingly, the extent to which bone structure is recovered post-weaning is site-dependent. Clinical studies observed that the recovery of bone mass is greater in the spine than long bones [1, 6, 7, 18]. Recent clinical and rodent studies demonstrated that maternal bone undergoes substantial structural adaptation to maintain the mechanical integrity of the high load-bearing skeletal regions, *e.g.*, trabecular compartment of the lumbar vertebrae and the cortical compartment of the long bone [18-25]. These findings suggested that physiological load-bearing may play an important role in regulating reproduction-induced skeletal alterations.

Despite the significant reduction in bone mass and mechanical properties, pregnancy- and lactation-associated fracture is uncommon [1]. Nevertheless, women with low bone mass or skeletal fragility prior to pregnancy may be predisposed to a higher risk of fractures. Given the importance of physiological load-bearing on regulating reproduction-induced skeletal changes, we asked the question: would external mechanical loading exert a protective and beneficial effect on bone health during reproduction, lactation, and post-weaning recovery? Although it is

suggested that physical exercise during pregnancy and breastfeeding benefits women's physical and psychological well-being [26], clinical investigations have reported variable results on the relationship between weight-bearing exercises and bone health during pregnancy and lactation. Some studies suggest that the physical activity is associated with a reduced extent of aBMD loss during pregnancy and lactation [27, 28], while others reported no benefits [29, 30]. An animal study using a bipedal stance model in rats found that weight-bearing exercise during pregnancy attenuated trabecular bone loss at the tibial metaphysis and increased cortical bone strength at the tibial diaphysis by the end of lactation [31]. Despite these previous data, further investigations are required to elucidate the detailed effects of mechanical loading on reproduction- and lactation-induced bone changes. Therefore, the first objective of this study was to investigate the maternal bone adaptations to external mechanical loading by utilizing an *in vivo* dynamic tibial loading protocol during pregnancy, lactation, and post-weaning recovery in a rat model. We hypothesized that external dynamic loading would attenuate the bone loss induced by pregnancy and lactation, and enhance the bone recovery post-weaning.

In addition, we also aimed to explore potential mechanisms through which the skeleton efficiently balances its metabolic and mechanical function during reproduction and lactation. As the long-lived primary mechanosensory cells in bone, the osteocytes likely play an important role in mediating skeletal mechano-responsiveness during reproduction and lactation. Osteocytes form an extensive cellular network through their numerous cell processes housed in the lacunar-canalicular system (LCS), an interconnected set of pores and channels embedded in the mineralized bone matrix. This LCS network allows nutrient supply, waste removal, and transport of signaling molecules [32, 33]. Moreover, loading-induced interstitial fluid flow inside the LCS transmits macroscopic mechanical stimuli to osteocytes through shear stress on the cell process

and fluid drag on the tethering elements that connect the cell processes with canalicular wall, triggering the release of various osteocytic signaling molecules that regulate mineral homeostasis [34]. LCS structure is thus a key determinant of fluid and solute transport that is essential for osteocyte signal transduction. Several studies in mice have demonstrated that, in various conditions, most notably during lactation, the LCS is modified through osteocyte perilacunar/canalicular remodeling (PLR) [35-41]. This lactation-induced PLR may alter the LCS porosity and the pericellular matrix (PCM) around osteocytes, thus affecting flow-mediated mechanosensing and diffusion/convection of nutrients and signaling molecules for osteocytes [13, 42]. Although the lactation-associated PLR has been demonstrated in mice, whether PLR occurs during lactation in rats still remains unclear. Therefore, the second objective of this study was to assess the effects of pregnancy, lactation, and post-weaning recovery on osteocyte microenvironment in a rat model. Based on the previous findings in mice, we hypothesized that osteocytes can actively remodel their perilacunar/canalicular bone matrix through PLR during lactation in rats, and this altered osteocyte microenvironment might be associated with altered mechano-responsiveness of post-partum bone.

2. Methods

2.1 Animal Protocol

All animal protocols were approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. Female, Sprague Dawley rats were assigned to 4 groups (n=10-11/group): Pregnancy, Lactation, Post-Weaning, and Virgin. Different timing of mating was chosen for each group so that all rats reached the same age of 26 weeks when loading experiments started (Figure 1). All rats received 2 weeks of dynamic loading. Rats in the Pregnancy group received dynamic loading from day 3.8 ± 2.2 to day 17.8 ± 2.2 of pregnancy. To

confirm the success of the pregnancy, euthanasia was delayed until parturition (4.2 days after the end of dynamic loading). Rats in the Lactation group underwent pregnancy followed by 14 days of lactation (loading initiated from day 1 of lactation) before euthanasia. Rats in the Post-Weaning group underwent pregnancy, 21 days of lactation, and 14 days of post-weaning recovery (loading initiated from day 1 post-weaning) before euthanasia.

To ensure sufficient dietary calcium content, all rats were fed a high-calcium diet (LabDiet 5001 Rodent Diet; 0.95% Ca). All litters were normalized to 9 pups per mother within 24 hours of birth for consistent lactation intensity. Throughout the experiment, all rats were housed in standard conditions of three rats per cage, except that rats in the reproductive groups were separated to one rat per cage during the last week of pregnancy and throughout the lactation period. All groups of rats were allowed to have normal cage activities throughout the experiment. Experiments were performed in three batches, performed 5 and 28 months apart, respectively (Supplementary Table S1). Two rats in the Virgin group were euthanized after the identification of mammary tumors, two rats in the Pregnancy group and one rat in the Lactation groups failed to become pregnant, one rat each in the Lactation and Post-Weaning groups died while giving birth, and one rat in the Lactation group as well as two rats in Post-Weaning groups died during the μ CT scans, resulting in a final sample size of $n=8$ for all groups.

2.2 In Vivo Dynamic Loading Protocol

A 2-week dynamic, compressive, uniaxial loading protocol for mouse tibia [43-45] was adapted for rats in this study. To determine the appropriate loading magnitude, a strain gauge study was performed on three separate sets of rat tibiae (methods and results described in the Supplementary Material): virgin rats at age of 4 months and 12 months, respectively, and 12-month-old reproductive rats that underwent two cycles of reproduction ($n=6-8$ tibiae/group).

Similar strain vs. load curves on the rat tibia midshaft were found for all three groups, indicating age and reproductive status did not significantly alter compliance of tibia to loading (Supplementary Figure S1 and Supplementary Table S4). Based on the strain gauge testing results, a pilot study of dynamic loading at a peak load of 36 N, corresponding to ~1,200 $\mu\epsilon$ at the tibia midshaft was conducted (n=3/group). Due to the lack of loading response at ~1,200 $\mu\epsilon$ detected by micro-computed tomography (μ CT) analysis and dynamic histomorphometry, a peak load of 45 N, corresponding to ~1,500 $\mu\epsilon$ at the tibia midshaft was chosen for the current study (Figure S1 and Table S4). Briefly, rats were anesthetized (4/2% isoflurane: 4% isoflurane for induction, and 2% isoflurane for maintenance), and a peak load of 45N was applied to the left tibiae at 2 Hz (*i.e.*, 0.15s ramp up, 0.15s ramp down, and 0.2s dwell time) by using a Bose Electroforce TestBench system (TA Instrument, Eden Prairie, MN, USA), for 5 minutes/session, 5 sessions/week over 2 weeks. The right tibia remained as a non-loaded, contralateral control. After each loading session, all rats were returned to their cages and allowed unrestricted activities. The weight of each rat was monitored during the loading period (Figure S2), and no rats were excluded from this study as the weight loss in all rats was less than 10% [45].

2.3 *In Vivo* μ CT Scans, Image Registration, and Bone Microstructural Analysis

Micro-computed tomography (μ CT) scans of both the right and left proximal tibia and tibial midshaft were performed right before (week 0) and at two weeks after the initiation of loading (week 2) using *in vivo* μ CT (Scanco vivaCT40, Scanco Medical AG, Brüttisellen, Switzerland), as described in our previously published study [46]. Briefly, rats were anesthetized (4/2% isoflurane), and one of the tibiae was fixed into a customized holder to ensure minimal motion. A 4.2 mm thick segment of the proximal tibia (located 0.3 mm below the proximal growth plate) and a 2.1 mm thick section of the tibial midshaft (located at the midpoint between

the end of the distal and proximal epiphysis) were scanned at 10.5 μm voxel size. The scanner was operated at 200 ms integration time, 145 μA current, and 55 kVp energy, and the average scan time was approximately 30 minutes per tibia. Rats were woken up and allowed to recover for ~3 hours from anesthesia between the two scans performed on each tibia.

To longitudinally track the changes in trabecular microstructure within a constant volume of interest (VOI), the sequential *in vivo* μCT scans at the proximal tibia were aligned, using a mutual-information-based, landmark-initialized, rigid registration procedure based on open source software (National Library of Medicine Insight Segmentation and Registration Toolkit) [46, 47]. A manually contoured, 1.5-mm thick trabecular VOI, located 2.6 mm distal to the growth plate (Figure S3A&S3B), was identified in the week 2 scan and registered back to the previous scan at week 0 based on the transformation matrices that resulted from the registration, and as a result, loading-induced changes in a consistent trabecular VOI of each rat were identified and monitored (Figure 2A). Standard parameters of trabecular bone microstructure [48] were measured within each trabecular VOI. Briefly, the μCT images of trabecular bone were Gaussian filtered (sigma=1.2, support=2) and thresholded by a global threshold (corresponding to 544 mgHA/cm³), identified through an adaptive threshold function provided by the μCT scanner manufacturer. Trabecular microstructural parameters, such as bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular spacing, (Tb.Sp), connectivity density (Conn.D), and structure model index (SMI) were quantified. For both non-loaded and loaded tibiae of each rat, the percent change from week 0 was computed for all trabecular parameters except SMI, which ranges between -3 to 3. Normalized SMI was computed by subtracting the SMI of week 0 from week 2 measurements for each rat.

To quantify changes in cortical structure, the scans of the tibial midshaft at week 2 were registered and rotated to align with the baseline scans. A 50-slice thick cortical VOI located at the center of the tibial midshaft (Figure S3A&S3C) was semi-automatically contoured using manufacturer-provided software (Scanco Medical AG, Brüttisellen, Switzerland). Voxels within the cortical VOI were then filtered (Gaussian filter, sigma=1.2, support=2), and thresholded by application of a global threshold corresponding to 822 mgHA/cm³. Standard parameters of cortical bone microstructure, including cortical area (Ct.Area), cortical thickness (Ct.Th), and polar moment of inertia (pMOI), were measured. The percent changes in cortical bone microstructural parameters between week 0 and week 2 were calculated in both non-loaded and loaded tibiae for each rat. Moreover, the corresponding loading-induced differences in cortical microstructural percent changes between loaded vs. non-loaded tibiae (% change in loaded tibia - % change in non-loaded tibia) for each rat were further calculated.

2.4 Dynamic Bone Histomorphometry on Periosteal Bone Formation

All rats received subcutaneous injections of calcein (20 mg/kg, Sigma-Aldrich, St. Louis, MO) at day 4 of loading, and intraperitoneal injections of alizarin complexone (30mg/kg, Sigma-Aldrich, St. Louis, MO) at day 13 of loading. These injection schedules corresponded to 11 and 2 days prior to euthanasia, respectively, except in Pregnancy group (14.8±2.2 and 5.8±2.2 days prior to euthanasia, respectively). After euthanasia, ~10 mm-long segments of midshafts of both tibiae were harvested, embedded in MMA, and cut into 0.5 mm-thick transverse sections using a low-speed diamond saw (IsoMet, Buehler). ApoTome equipped Zeiss Axio Zoom.V16 microscope was applied to for image capture and subsequent digital signal correction, and the dynamic bone histomorphometry was performed to assess bone formation rate (BFR/BS), mineral apposition rate (MAR), and mineralizing surfaces (MS/BS) at the periosteal surface of

the tibial midshaft using OsteoMeasure Software (OsteoMetrics, Inc., Decatur, GA), where BS is the entire periosteal circumference [49]. The fold changes of bone formation parameters between loaded vs. non-loaded tibiae for each rat ((Bone formation parameters in loaded tibia - non-loaded tibia)/ non-loaded tibia) were calculated to determine the loading-induced bone formation. For samples with no double-labeled surface, the average value of MAR for the cohort to which the sample belongs was assigned for its MAR and was used to calculate BFR/BS [49]. The number of animals that had missing double-labeled surface was shown in Supplementary Table S2.

2.5 Ploton Silver Staining on Osteocyte Lacunar-Canalicular Network in the Non-loaded tibia

Immediately after euthanasia, ~15 mm-long segments of the left (non-loaded) distal tibiae starting from 2 mm proximal to the distal tibial-fibular junction were harvested, fixed in 4% paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS) for 48 hours, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 30 days, and then embedded in paraffin. 8 μ m-thick longitudinal paraffin sections were prepared for the non-loaded tibiae collected from the first and second batches of 45N loading study (n=4-6/group) and the 36N loading study (n=0-2/group), making a final sample number of 6 animals per group (details shown in Supplementary Table S3).

To assess changes in lacunar-canalicular network structure, Ploton silver staining was performed on paraffin sections as previously described [40, 50]. Briefly, the de-paraffined sections were incubated in freshly mixed working silver nitrate solution, consisting of two parts of 50% silver nitrate and one part 1% formic acid in 2% gelatin (Type B gelatin from bovine skin, Sigma) solution, for 55 mins, washed in 5% sodium thiosulfate for 10 mins, and then dehydrated and mounted. Thirty lacunae were randomly selected from both medial and lateral

sides (n=15/side) on a 0.5 mm-long region of the tibial cortex located at 1 mm proximal to the distal tibial-fibular junction (n=6 rats/group) to quantify lacunar area, lacunar perimeter, the number of canaliculi emanating from each lacuna, and the number of canaliculi per lacunar surface.

2.6 Bone Immunohistochemistry (IHC) on Osteocyte PLR Activities in the Non-loaded Tibia

To assess PLR enzyme activities, paraffin sections were incubated with primary antibodies for MMP13 (1:100; Abcam, ab39012) or CtsK (1:75; Abcam, ab19027), followed by incubations with corresponding biotinylated secondary antibody, avidin-conjugated peroxidase, and diaminobenzidine substrate chromogen system (Innovex Universal Animal IHC kit) to detect positively stained cells [51]. Sections stained with Rabbit sera IgG were used as negative controls (Figure S4). All measurements were made within a 0.5-mm long region of both medial and lateral tibial diaphysis located at 1 mm proximal to the distal tibial-fibular junction. For quantification, each lacuna was manually scored as either positive or negative using OsteoMeasure Software (OsteoMetrics, Inc., Decatur, GA). Subsequently, the percentage of positively stained lacunae was determined.

2.7 Statistical Analysis

All results are reported as mean $\pm$ standard deviation. Comparisons of loading-induced changes in the same animal, *e.g.* week 0 (pre-loading) vs. week 2 (post-loading), as well as left (loaded) vs. right (non-loaded) tibiae, were made using student's paired t-test; while one-way ANOVA with Bonferroni corrections was used to compare loading responses and osteocyte-related parameters in rats with different reproductive status. For all tests, p-values below 0.05 were considered statistically significant, while p-values below 0.1 were considered as a trend of difference with specific p-value provided.

3. Results

3.1 Effects of reproduction and lactation on trabecular and cortical bone microstructure at the non-loaded tibia

For the non-loaded tibiae, Virgin rats underwent minimal changes in all trabecular parameters over the 2-week period (Figure 2). In contrast, non-loaded tibiae in Pregnancy rats underwent a trend of 6% reduction in BV/TV ($p=0.058$), 4% and 9% reductions in Tb.N and Conn.D, respectively and a 4% increase in Tb.Sp over 2 weeks (Figure 2B-F). Moreover, for the non-loaded tibiae of Lactation rats, BV/TV, Tb.Th, Tb.N, and Conn.D dropped 20-81%, Tb.Sp increased by 87% over the 2 weeks, and SMI was 0.69 above the level at parturition (week 0, Figure 2B-G). Following weaning, the trabecular microstructure partially recovered, compared to the end of lactation. At 2 weeks post-weaning, BV/TV, Tb.Th, Tb.N, and Conn.D of non-loaded tibiae of the Post-Weaning group were elevated by 116%, 31%, 12%, and 169%, respectively, while Tb.Sp decreased by 11% and SMI lowered by 0.30 when compared to the end of lactation (week 0, Figure 2B-G). However, all trabecular bone microstructural parameters of the Post-Weaning rats (week 2) were still significantly lower than those in the age-matched virgins (Supplementary Figure S2).

In contrast to the substantial changes in trabecular bone at the proximal tibia, reproduction and lactation induced minimal alterations in cortical bone structure at the tibial midshaft (Figure 3A-C). As a result, no difference was found in any cortical bone parameters of the non-loaded tibia among groups (Figure S2). Over a 2-week period, Virgin rats underwent 0.5% and 1% increases in Ct.Area and pMOI, respectively. Moreover, Ct.Area, pMOI, and Ct.Th increased 1.5%, 2.5%, and 1.3%, respectively, over 2 weeks of pregnancy. No change was observed in Lactation rats except a trend of 1.4% decrease in Ct.Area ($p=0.09$) over 2 weeks of

lactation, while trends of increases of 1.2%, 0.6%, and 0.6% in Ct.Area ($p=0.07$), pMOI ($p=0.097$), and Ct.Th ($p=0.07$), respectively, were detected in Post-Weaning rats over the 2 weeks post-weaning.

3.2 Loading responses in trabecular bone microstructure at the proximal tibia

Two weeks of dynamic loading induced minimal loading responses in trabecular bone in Virgin, Pregnancy, and Post-Weaning groups (Figure 2). For virgin rats, no loading response was observed in trabecular bone except a 2% greater increase in Tb.Th in the loaded vs. non-loaded tibiae. For rats in the Pregnancy group, loading led to a greater increase in SMI in the loaded vs. non-loaded tibia, indicating a greater deterioration of plate-like trabeculae. Moreover, for rats in the Post-Weaning group, loading led to a trend toward reduced recovery in Conn.D. In contrast, loading reduced trabecular bone deterioration in the Lactation group (Figure 2). Although the loaded tibiae in Lactation rats still experienced a reduction in BV/TV, this bone loss was significantly attenuated by 11% when compared to the non-loaded tibiae (Figure 2B). Moreover, loading completely abolished the lactation-induced reduction in Tb.Th and attenuated the loss in Conn.D in Lactation rats (Figure 2C&2F).

3.3 Loading responses in cortical bone structure at the tibial midshaft

Significant loading responses were found in cortical bone structural parameters in all groups of rats (Figure 3A-C). The 2-week loading elevated Ct.Area, pMOI, and Ct.Th by 2.9%, 4.9%, and 2.3%, respectively, in Virgins, which were significantly greater than changes in the non-loaded tibia. Loading further enhanced the increases of Ct.Area and pMOI in the Pregnancy group to 3.8% and 6.0%. Moreover, the loading process halted the trend of lactation-induced deterioration in cortical bone structure, and further induced a 3.7% increase in pMOI. Following weaning, Ct.Area, pMOI, and Ct.Th increased by 3.6%, 6.5%, and 2.8% in the loaded tibia in

the Post-Weaning group while no change was detected in the non-loaded tibia. To further compare the extent of loading responses among groups, the differences in percent change in cortical bone structure between the loaded and non-loaded tibia were calculated for each rat (Figure 3D-F). However, no difference was found in the degree of loading responses in any cortical bone structural parameters among groups (Figure 3D-F).

3.4 Loading responses in cortical periosteal bone formation at the tibial midshaft

After two weeks of dynamic loading, MS/BS, MAR, and BFR/BS were significantly elevated in the loaded vs. non-loaded tibia in all groups (Figure 4A-D). To further compare the extent of loading responses among different groups, the fold changes in bone formation parameters between the loaded and non-loaded tibiae were derived. Intriguingly, Lactation rats had a higher fold change in MS/BS than Pregnancy and a trend towards greater fold change than Virgin rats (Figure 4E). Moreover, fold change in MAR was greater in Lactation group compared to Pregnancy and Post-Weaning groups (Figure 4F), and as a result, Lactation rats had the highest loading-induced fold change in BFR/BS among all groups (Figure 4G).

3.5 Effects of reproduction and lactation on osteocyte lacunar-canalicular network structure and PLR activities

Qualitatively, no distinct differences in the lacunar-canalicular network were observed among groups (Figure 5A). Quantitative comparisons indicated that the lacunar perimeter was 10%, 7%, and 8% greater in Lactation rats when compared to Virgin, Pregnancy, and Post-Weaning groups, respectively (Figure 5B). Similarly, Lactation rats had 15-21% greater lacunar area than all other groups (Figure 4C). No difference was detected in the number of canaliculi emanating from one lacuna or in the number of canaliculi per lacunar surface (Figure 5D&5E).

Significant PLR enzymatic activities were detected in Lactation rats. The percentage of MMP13-positive osteocytes was 37% and 40% greater in Lactation than Virgin and Post-Weaning rats, respectively (Figure 6A&6B). Similarly, percentage of osteocytes that stained positive for CtsK was 50% higher in Lactation than Post-Weaning rats (Figure 6C&6D).

4. Discussion

This study was the first investigation on the effects of dynamic loading on bone health during pregnancy, lactation, and post-weaning recovery in an *in vivo* animal model. By applying a dynamic tibial loading protocol with 1,500 $\mu\epsilon$ at tibial midshaft to mimic jogging or running in women, we demonstrated that despite the minimal loading responses detected at the trabecular bone in virgins, loading reduced lactation-induced trabecular bone loss and improved cortical bone structure regardless of reproductive status, indicating a beneficial effect of weight-bearing exercise on maternal bone during pregnancy and postpartum. Moreover, loading led to greater periosteal bone formation in lactating rats compared to rats at other reproductive stages or age-matched virgins, indicating a greater mechano-responsiveness of maternal bone during lactation.

Variable results were reported in clinical investigations regarding the effect of weight-bearing activities during pregnancy and post-partum [27-30]. Discrepancies may be due in part to inconsistencies in voluntary exercise intensity and differences in the stage of reproduction and lactation among studies. By applying dynamic loading to rats at well-defined reproductive stages, the current study directly evaluated the potential of load-bearing exercise to modulate bone responses to reproduction and lactation. We applied a 2-week dynamic tibial compressive loading that induced a peak of $\sim 1,500 \mu\epsilon$ near the lateral mid-shaft surface, which mimics jogging or running in women [52, 53]. Minimal loading responses were observed in trabecular bone at the proximal tibia in virgin, pregnancy and post-weaning rats, while a significant loading

response was detected in trabecular bone of lactation rats, indicating a loading-induced attenuation in trabecular bone loss during lactation. However, despite attenuated trabecular bone loss during lactation, the absolute values of trabecular microstructural parameters in Lactation rats were still lower than the age-matched virgins. In contrast, dynamic loading enhanced cortical bone structure and periosteal bone formation in all groups, suggesting that weight-bearing exercise at moderate intensity exerts a protective effect or enhancement on cortical bone in rats regardless of their reproductive stage. Furthermore, intriguingly, the loading-induced increase in bone formation rate at the periosteal cortex of the tibial midshaft in lactating rats was greater than that observed in virgins or other reproductive groups, indicating a greater mechanoresponsiveness of cortical bone during the lactation period. Consistent with our findings, a previous study conducted *ex vivo* loading on the mouse fibula, and found a stronger loading-induced reduction in osteocytic sclerostin expression as well as a greater elevation in β -catenin expression by osteocytes in lactating mice than age-matched virgins, suggesting that the osteocytes in fibulae from lactation mice may have a stronger response to mechanical loading [54]. The enhanced loading responses in both trabecular and cortical bone during the lactation period further emphasize the beneficial effects of physical exercises on maternal bone health postpartum.

Our longitudinal μ CT imaging approach allowed for the tracking of local changes in the trabecular and cortical bone within a constant volume of interest (VOI) over the 2-week period during pregnancy, lactation, and post-weaning recovery in both loaded and non-loaded tibiae. As expected, reproduction and lactation induced substantial alterations in trabecular bone microstructure at the proximal tibia, which aligns with several previously published studies [14, 22, 23]. For the first time, structural changes at the tibial midshaft cortex were monitored by

using *in vivo* μ CT imaging. During pregnancy, a small but significant increase in cortical bone size at the tibia midshaft was detected. This is similar to what has been reported in the tibial metaphysis, where the tibial cortex significantly increased, compensating the trabecular bone deterioration during pregnancy [22]. Our results also indicated a trend of reduction in cortical bone parameters at the tibial midshaft during lactation. In agreement with our findings, several rodent studies suggested that the adverse effects of lactation are less pronounced at the predominantly cortical tibial and femoral midshaft [16, 21, 55, 56]. These μ CT-based findings were further confirmed by the results of our dynamic histomorphometry analysis, which align well with previous findings that the periosteal bone formation was elevated during pregnancy, and ceased during lactation [17]. Following weaning, only trends of tiny increases in tibial midshaft cortex was detected in the current study. This is consistent with previous investigations in femoral midshaft of rats and mice, which suggested that the cortical bone structure still remains deteriorated post-weaning when compared to virgins [16, 21, 25, 57, 58]. Nevertheless, previous studies suggested that improvements in intrinsic tissue mechanical properties may compensate the cortical bone structural deficit, resulting in no difference in femoral stiffness or peak load between post-weaning and virgin bone [24, 25]. Taken together, at the critical load-bearing sites such as the tibia or femur midshaft, the significant improvement in cortical bone structure during pregnancy, less pronounced reduction at cortical bone during lactation, and further improvements in material properties after weaning may work together to offset the potential loss of strength induced by trabecular bone deficit following reproduction and lactation.

Consistent with previous studies in CD1 and C57BL/6 mice [35, 36, 39, 40, 59], our study further confirmed that lactation resulted in increased lacunar size and osteocytic PLR enzyme activities in rats, and these transient changes were reverted post-weaning. No significant

changes in lacunar morphology were observed during pregnancy. Moreover, no striking changes in canalicular network were observed throughout the whole reproductive cycle, which agrees with previous findings in mice [39]. In contrast to our findings, a previous study evaluating lacunar diameter using μ CT with a voxel size of 2 μ m found no difference between lactation and virgin rats on normal diet [58]. Given the small size of lacunae (~ 10 μ m in the short axis [34]) and the subtle change during lactation (7~10% increase in perimeter), the limited image resolution of μ CT may not allow the detection of such small changes in LCS induced by lactation. Another study using Naval Medical Research Institute (NMRI) mice also reported no lactation-associated changes in lacunar size and morphology, suggesting that the effects of lactation on lacunar structure may be strain-dependent in rodents [60].

The total surface area of osteocyte lacunae has been estimated to be several magnitudes larger than that of the trabecular bone surfaces upon which osteoclasts can act, and as a result, small alterations in osteocyte lacunae and canaliculi through PLR during lactation may lead to a substantial change in bone mineral content at the level of the whole animal [39, 61]. Additionally, the LCS structure, particularly the pericellular matrix (PCM) in the LCS, also plays a critical role in osteocyte mechano-transduction. It has been suggested that the interstitial fluid flow in the LCS, driven by external mechanical loading, may amplify tissue-level strains for osteocytes to sense and respond to [34, 62, 63]. Through PLR, the lactation-associated changes in LCS and its PCM may have an impact on the mechanical signals perceived by osteocytes and their cell processes, leading to altered bone mechano-responsiveness. To understand the potential impact of PLR on osteocyte mechanosensing, Lai *et al.* [42] quantified and compared the loading-induced fluid flow, flow-mediated mechanical stimulation on osteocytes, and solute transport in LCS in the lactating and nulliparous skeleton using a multiscale modeling approach

built upon the LCS fluid flow model established by Weinbaum *et al.* [64] and experimental and modeling system developed by Wang *et al.* [43, 63, 65]. Using the key LCS features based on published experimental data of lactating and nulliparous murine tibiae, the model predicted that increased LCS porosity alone would lead to a marked increase of fluid drag force on the osteocyte body and cell processes under a high frequency (4Hz) loading. Furthermore, the increased LCS dimensions will lead to increased transport flux of signaling molecules, nutrients, and waste products among osteocytes and between osteocytes and cells residing on the bone surface. Moreover, degradation of the PCM fibers, which most possibly would occur in matrix lysis [35, 37, 66], along with high frequency loading, would synergistically promote flow-mediated mechanical stimulations on osteocytes and their cell processes, and enhance solute transport in the LCS. Based on these modeling results, we hypothesized that lactation-induced structural changes of the LCS and its PCM alter the microenvironment of osteocytes, leading to enhanced osteocyte mechanosensing. Aligning with our working hypothesis, osteocyte lacunae in aging mice are smaller and more spherical, with reduced PCM density [43, 67], which has been suggested to be associated with the reduced bone mechano-responsiveness with aging [68, 69]. Further studies are warranted to explore the effects of reproduction and lactation on osteocyte LCS ultrastructure, pericellular space and fiber density, and to experimentally establish their direct influences on the load-induced, fluid-mediated mechanical signals on osteocytes and other bone cells.

In addition to the positive effects of dynamic loading during pregnancy and post-partum on maternal bone, the mother's participation in physical activities may affect the offspring. Although we did not investigate the effects of dynamic loading during pregnancy and lactation on fetal/infant development in our current study, no obvious difference in the size of neonatal

pups or subsequent growth rate of pups was observed throughout the experiment when compared to the “normal litters” from mothers with standard cage activities. Intriguing, a recent study recruited 141 women and categorized them into different activity groups based on activity types and intensities, and found no relationship between physical activity during pregnancy and neonatal birth weight [70]. Another recent study showed that increased levels of physical activity by pregnant women are associated with improved breastfeeding outcomes, which may benefit their offspring [71]. Moreover, it has been suggested that moderate exercise does not affect milk supply, milk macronutrient and mineral composition, or infant growth [72]. Taken together, a moderate level of dynamic loading during pregnancy and postpartum appears to exert no adverse effects on the offspring.

Our study was not without limitations. Firstly, the effects of reproduction and lactation on osteocyte LCS network and PLR activities were not examined in the loaded tibiae. Our study was designed with the primary objective of investigating the loading responses in rats with different reproductive status, while the secondary objective was to examine the alterations of lacunar-canalicular system (LCS) over the reproductive cycle. Therefore, the proximal tibiae and tibial midshafts were embedded in MMA to assess loading responses, while only the distal tibiae were harvested for paraffin embedding to assess LCS network and PLR activities. Previous studies have suggested that the strain level experienced at rat distal tibia is much lower compared with the tibial midshaft, indicating that the distal tibia is not an ideal location to analyze loading effects [73]. Moreover, a recent study found that the loading process with 700-1,200 $\mu\epsilon$ peak led to 20% increase in canalicular length and elevated PLR mRNA level at the proximal tibia, but no loading-induced difference was detected in the distal tibia, indicating that the mechanoregulation of PLR was site-specific [74]. Therefore, future studies that are designed to directly assess PLR

476 at the proximal tibia, are warranted to investigate the loading-induced effects on LCS network
477 and PLR activities. Secondly, the ideal strain gauge measurements to identify loading magnitude
478 should be conducted in rats with the same age of 7 months, however, the current strain gauging
479 study was performed in rats with different ages and reproductive status, including 4-month-old
480 virgin female rats, 12-month-old virgin female rats, and 12-month-old reproductive rats (n=6-8
481 tibiae/group). Similar strain-load curves were observed in all groups of female rats, indicating
482 that age and reproductive status did not significantly alter compliance of tibia to loading, and
483 thus, we expect that the tibia of rats at age of 7 months would share a similar strain-load
484 relationship with those of other female groups. Moreover, the maternal trabecular bone
485 underwent rapid changes during pregnancy, lactation, and post-weaning recovery, making it
486 difficult to adjust the peak load to achieve consistent tissue strains over the 2-week loading
487 period. Therefore, a 45N peak load, corresponding to $\sim 1,500 \mu\epsilon$ on the tibial midshaft surface in
488 virgin rats, was applied to all reproductive groups. However, reduced trabecular bone volume
489 and deteriorated microstructure in the Lactation and Post-Weaning rats may lead to different
490 strain distributions inside the trabecular bone compartment when compared to Virgin and
491 Pregnancy groups. Nevertheless, the majority of the load applied to long bone is carried by the
492 cortical bone compartment. In the current study, no difference was found in the tibial midshaft
493 structure among groups (Figure S2). Therefore, we expect the average tissue strains applied to
494 the tibial midshaft to be comparable among groups. To further derive and compare the mechano-
495 sensitivity of maternal bone at different reproductive stages, additional pilot experiments with
496 peak load at $\sim 1,200$ and $\sim 1,800 \mu\epsilon$ surface strain were conducted (data not shown). However,
497 while minimal loading responses were observed at $\sim 1,200 \mu\epsilon$ (36N), the loading experiment at
498 $\sim 1,800 \mu\epsilon$ was terminated early due to the soft tissue damage in the rat joint caused by the high

peak load (54N). To overcome this limitation, a mouse model will be established in our future studies, where a greater range of strains of 1,200-2,000 $\mu\epsilon$ on the medial bone surface can be safely applied to induce robust adaptive loading responses on the tibia [45].

Despite these shortcomings, our study provided a thorough evaluation of the effects of pregnancy, lactation, and weaning on maternal bone mechano-responsiveness and osteocyte microenvironment in rats. By applying a dynamic loading protocol, we demonstrated the beneficial effect of loading on enhancing cortical bone structure of maternal bone during pregnancy, lactation, and post-weaning recovery. Particularly, maternal bone during lactation exhibited a greater mechano-responsiveness to dynamic loading in both trabecular and cortical bone compartments when compared to Virgin, Pregnancy, and Post-Weaning rats. Furthermore, elevated osteocyte PLR activities during lactation resulted in significantly increased osteocyte lacunar dimensions in lactating rats. This may lead to changes in interstitial fluid flow-mediated mechanical stimulation on osteocytes and increased solute transport in the LCS during high-frequency dynamic loading, thus enhancing mechano-responsiveness of maternal bone during lactation. Taken together, this study provides important insight into the relationship between reproduction and lactation-induced skeletal changes and external mechanical loading, emphasizing the importance of weight-bearing exercise on maternal bone health postpartum.

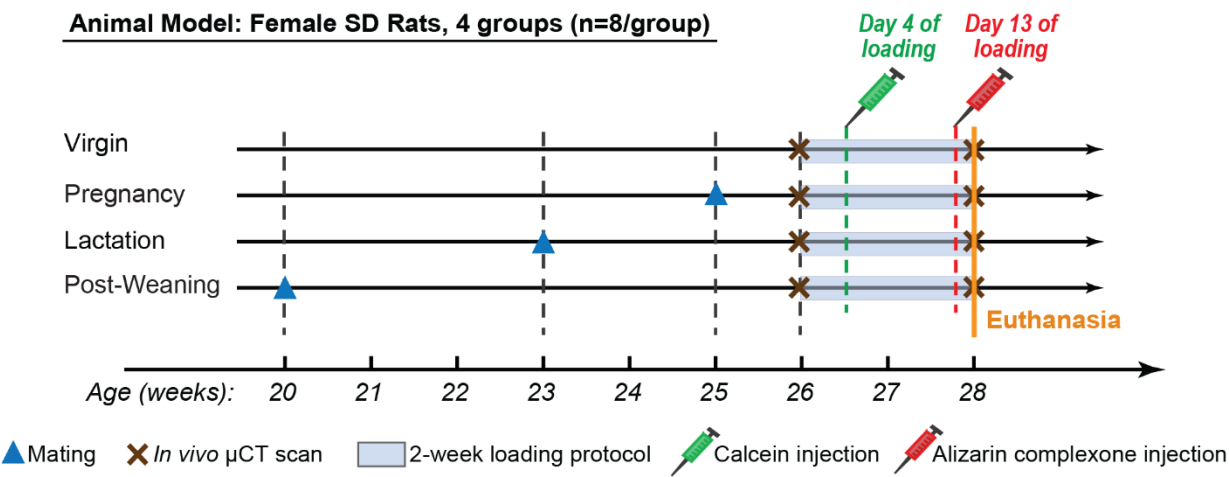
5. Acknowledgements

We thank Dr. Tamara Alliston and Dr. Cristal Yee, University of California, San Francisco, for kindly providing the protocols of Ploton silver staining and immunohistochemistry of MMP13 and CtsK.

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522 (to XSL), NIH/NIAMS F32-AR076906 (to RC), and National Science Foundation (NSF) Award
523 #1653216 (to XSL).

524 **Figures:**



525
526 **Figure 1.** Schematics of study design.

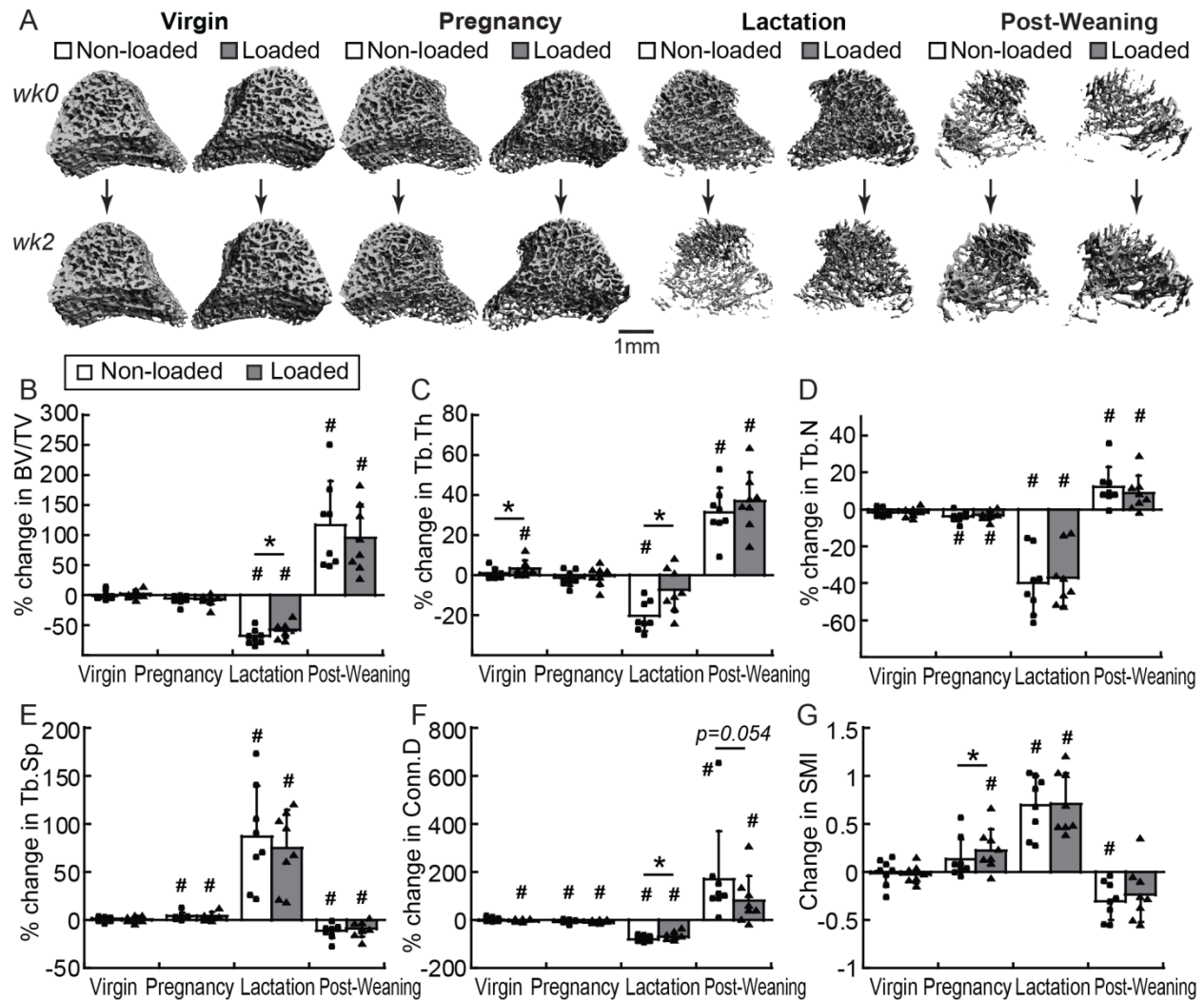


Figure 2. (A) Representative 3D renderings of trabecular bone at the proximal tibia in Virgin, Pregnancy, Lactation, and Post-Weaning rats. % changes in (B) BV/TV, (C) Tb.Th, (D) Tb.N, (E) Tb.Sp, and (F) Conn.D, as well as (G) change in SMI in loaded and non-loaded tibiae in rats with different reproductive status. $p < 0.05$: # indicates significant changes over the 2-week time period (week 0 vs. week 2); * indicates significant differences between loaded and non-loaded tibiae.

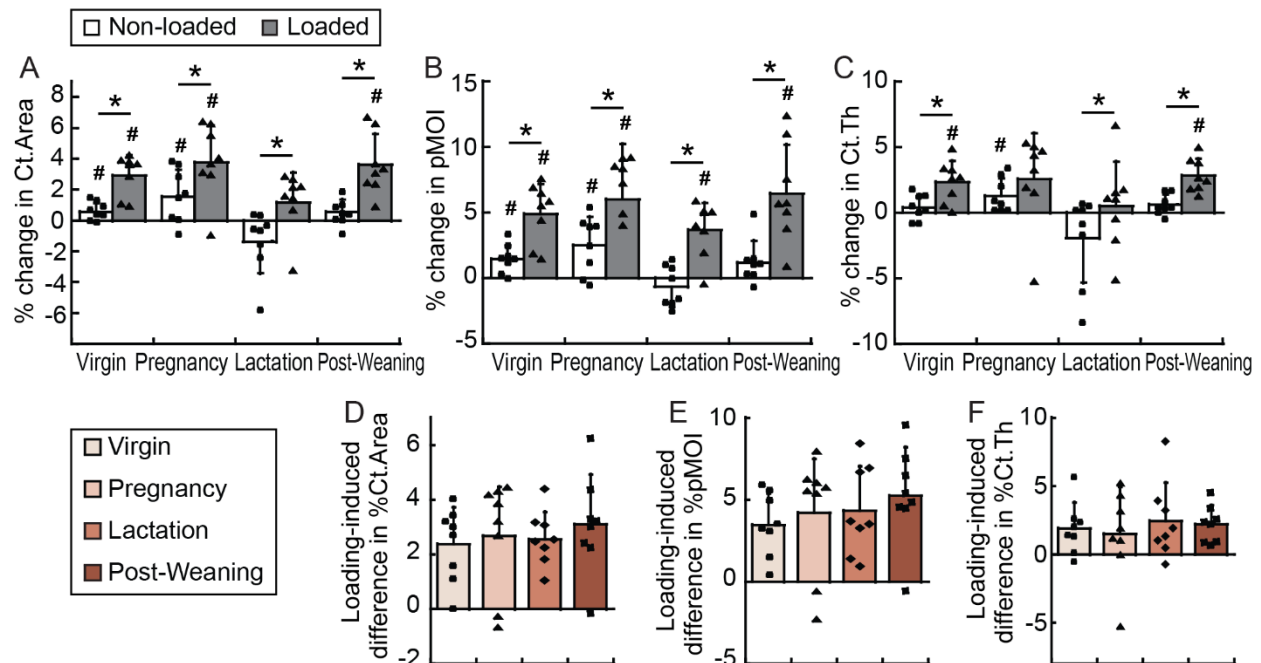


Figure 3. % changes in (A) Ct.Area, (B) pMOI, and (C) Ct.Th at tibial midshaft in loaded and non-loaded tibiae in rats with different reproductive status. Comparisons of loading-induced differences in % change between loaded and non-loaded tibiae in (D) Ct.Area, (E) pMOI, and (F) Ct.Th at tibial midshaft in rats with different reproductive status. $p < 0.05$: # indicates significant changes over the 2-week time period (week 0 vs. week 2); * indicates significant differences between loaded and non-loaded tibiae.

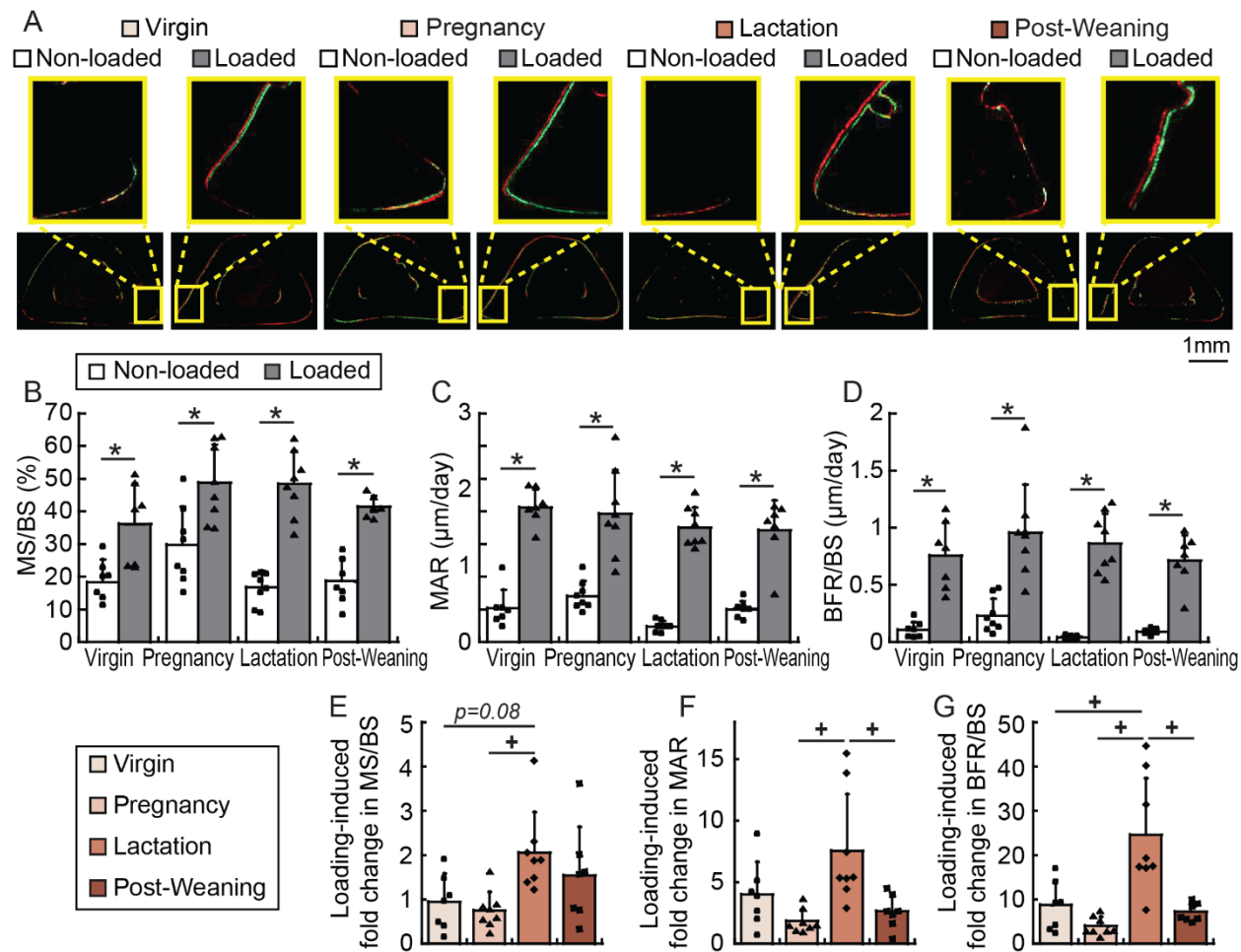


Figure 4. (A) Representative histology images of tibial midshaft. Comparisons of (B) MS/BS, (C) MAR, and (D) BFR/BS at periosteal cortical bone of tibial midshaft between loaded and non-loaded tibiae in rats with different reproductive status. Comparisons of loading-induced fold changes in periosteal (E) MS/BS, (F) MAR, and (G) BFR/BS at tibial midshaft in rats with different reproductive status. $p < 0.05$: * indicates significant differences between loaded and non-loaded tibiae; + indicates significant differences in loading-induced fold change among groups.

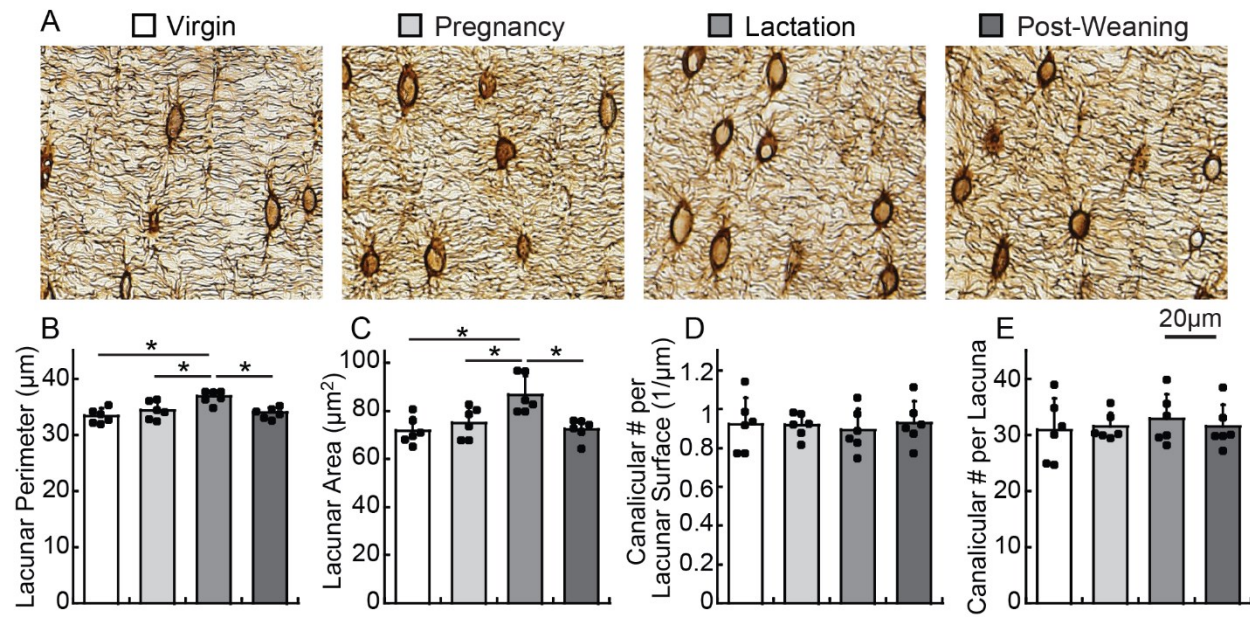


Figure 5. (A) Representative images of lacunar-canalicular network by Ploton silver staining. Comparisons of (B) lacunar perimeter, (C) lacunar area, (D) canalicular number per lacunar surface, and (E) canalicular number per lacuna at tibial cortex in rats with different reproductive status. * indicates significant differences among groups ($p<0.05$).

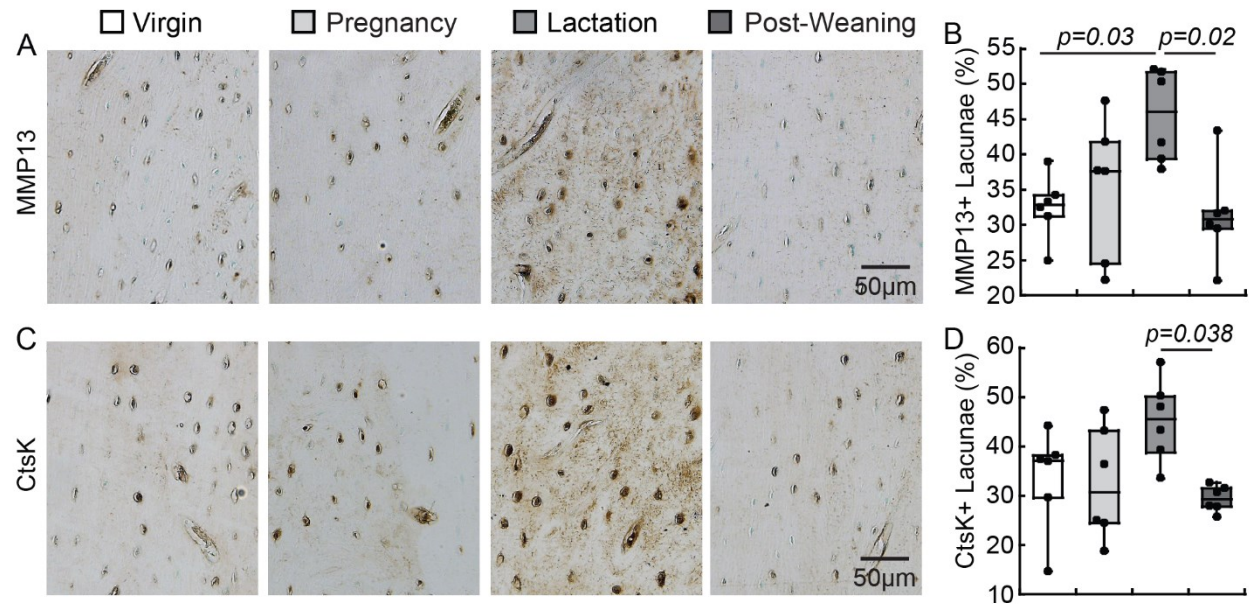


Figure 6. Representative images of (A) MMP13 and (C) CtsK immunostaining. Comparisons of (B) % MMP13-positive and (D) CtsK-positive osteocytes at tibial cortex in rats with different reproductive status. * indicates significant differences among groups ($p<0.05$).

Supplementary Materials

Methods of strain gauge study:

A separate set of rats, including 4-month-old male rats, 4-month-old virgin female rats, 12-month-old virgin female rats, and 12-month-old reproductive rats, were utilized for *ex vivo* strain gauging experiment to determine the required load magnitude (n=6-8 tibiae/group) of the dynamic compressive loading protocol. Reproductive rats underwent 2 cycles of pregnancy, with each cycle consisting of a 3-week lactation and a 6-week post-weaning recovery period, followed by euthanasia 8 weeks after the end of the 2nd cycle. A single-element gauge (EA-06-015DJ-120; Measurements Group, Inc., Raleigh, NC) was attached on the relatively flat medial surface (1-2 mm proximal of tibial mid-diaphysis) of the intact tibia that remained attached to the rat body. A compressive loading with gradually increased magnitude (0.5N/s to 60N) was applied to the tibia by utilizing a Bose Electroforce TestBench® loading system (TA Instrument, Eden Prairie, MN, USA), and the corresponding output voltage of the strain gauge was automatically recorded by LM1's data acquisition unit and converted to strain by applying a calibrated conversion coefficient. The compressive rigidity of tibiae for each group of rats was calculated from the strain-load curves derived from the loading tests. To achieve a consistent 1500 $\mu\epsilon$ strain on the tibial surface, the loading magnitudes for the four groups of rats were calculated to be 55.8 $\pm$ 3.4 N, 44.0 $\pm$ 3.2 N, 44.9 $\pm$ 4.2N, and 44.7 $\pm$ 7.0N for 4-month-old males, 4-month-old virgin females, 12-month-old virgin females, and 12-month-old reproductive females, respectively (Table S4). Based on the similar strain-load curves observed in all groups of female rats regardless of age or reproductive status (Fig S1), we expect that the tibia of rats at age of 7 months would share a similar strain-load relationship with those of other female groups. In our pilot study, a peak load of 36N (corresponding to $\sim$ 1200 $\mu\epsilon$ on the surface of tibial midshaft) was

583 applied to the 7-month-old female rats, but minimal loading responses were detected (data not
584 shown). Therefore, a loading magnitude of 45N, which corresponds to a higher strain of ~1500
585 $\mu\epsilon$ on the tibial midshaft surface, was utilized to induce adaptive loading responses in the current
586 study in all groups of rats.

Table S1: Number of rats per group from each batch

Group	Animal number in each batch (n)		
	<i>Batch 1</i>	<i>Batch 2</i>	<i>Batch 3</i>
Virgin	3	5	2
Pregnancy	3	3	4
Lactation	3	4	4
Post-Weaning	3	4	4

Table S2: Number of rats with missing double-labeled surface in each group

Group	Number of rats with missing double-labeled surface
Virgin	2
Pregnancy	1
Lactation	4
Post-Weaning	2

Table S3: Sample number of non-loaded tibia in each group for immunohistomorphometry
from pilot (36N) and main (45N) studies

Group	Sample number of non-loaded tibia in each study (n)	
	<i>Pilot study (36N)</i>	<i>Main study (45N)</i>
Virgin	0	6
Pregnancy	2	4
Lactation	1	5
Post-Weaning	1	5

591 **Table S4:** Relationships between strains ($\mu\epsilon$) on the surface of tibial midshaft and the
592 corresponding magnitude of compressive load required (N)

Strains ($\mu\epsilon$)	Magnitude of Compressive Load Required (N) *			
	<i>4mo Male</i>	<i>4mo Virgin Female</i>	<i>12mo Virgin Female</i>	<i>12mo Reproductive Female</i>
500	18.6 $\pm$ 1.1	14.7 $\pm$ 1.1	15.0 $\pm$ 1.4	15.0 $\pm$ 2.3
750	27.9 $\pm$ 1.6	22.0 $\pm$ 1.6	22.5 $\pm$ 2.1	22.4 $\pm$ 3.5
1000	37.2 $\pm$ 2.2	29.3 $\pm$ 2.1	29.9 $\pm$ 2.8	29.8 $\pm$ 4.7
1250	46.5 $\pm$ 2.8	36.6 $\pm$ 2.7	37.4 $\pm$ 3.5	37.3 $\pm$ 5.9
1500	55.8 $\pm$ 3.4	44.0 $\pm$ 3.2	44.9 $\pm$ 4.2	44.7 $\pm$ 7.0
1800	67.0 $\pm$ 4.1	52.7 $\pm$ 3.8	53.9 $\pm$ 5.0	53.7 $\pm$ 8.4

593 *: Data represented as mean $\pm$ SD.

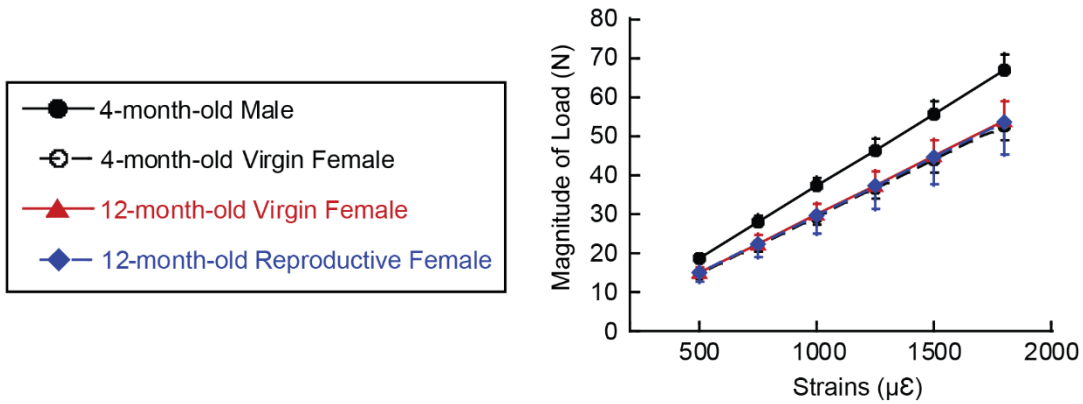
594 **Table S5:** Trabecular and cortical bone microstructural parameters at week 2 in non-loaded and
595 loaded tibiae in rats with different reproductive status

Structural Parameters (at week 2)	Virgin		Pregnancy		Lactation		Post-Weaning	
	<i>Non-loaded</i>	<i>Loaded</i>	<i>Non-loaded</i>	<i>Loaded</i>	<i>Non-loaded</i>	<i>Loaded</i>	<i>Non-loaded</i>	<i>Loaded</i>
<i>Proximal tibia</i>								
BV/TV	0.28 ± 0.09	0.29 ± 0.08	0.26 ± 0.05	0.27 ± 0.04	0.05 ± 0.03	0.07 ± 0.03*	0.08 ± 0.05	0.10 ± 0.06*
Tb.Th (mm)	0.071 ± 0.008	0.072 ± 0.008*	0.067 ± 0.004	0.067 ± 0.003	0.047 ± 0.004	0.054 ± 0.006*	0.060 ± 0.010	0.067 ± 0.008*
Tb.N (1/mm)	5.39 ± 1.07	5.16 ± 0.92	5.37 ± 0.84	5.26 ± 0.86	2.80 ± 0.82	2.89 ± 1.04	2.80 ± 0.82	2.87 ± 0.77
Tb.Sp (mm)	0.17 ± 0.03	0.18 ± 0.03	0.17 ± 0.03	0.17 ± 0.03	0.43 ± 0.21	0.39 ± 0.15	0.39 ± 0.16	0.38 ± 0.15
Conn.D (1/mm ³)	166.8 ± 64.2	158.8 ± 52.3	166.8 ± 64.2	168.8 ± 64.5	22.1 ± 23.1	33.9 ± 29.6	28.1 ± 21.8	34.8 ± 23.5
SMI	1.54 ± 0.32	1.14 ± 0.61*	1.54 ± 0.32	1.45 ± 0.21	2.99 ± 0.26	2.90 ± 0.23	2.76 ± 0.49	2.87 ± 0.57
<i>Tibial midshaft</i>								
pMOI (mm ⁴)	5.23 ± 0.86	5.60 ± 1.07*	5.50 ± 0.80	6.00 ± 0.86*	5.22 ± 0.64	5.79 ± 0.73*	5.52 ± 1.16	6.23 ± 1.26*
Ct.Area (mm ²)	4.18 ± 0.35	4.37 ± 0.36*	4.29 ± 0.25	4.46 ± 0.23*	4.18 ± 0.18	4.39 ± 0.22*	4.22 ± 0.43	4.47 ± 0.41*
Ct.Th (mm)	0.58 ± 0.03	0.60 ± 0.02*	0.59 ± 0.03	0.6 ± 0.02	0.58 ± 0.03	0.59 ± 0.02*	0.57 ± 0.03	0.59 ± 0.03*

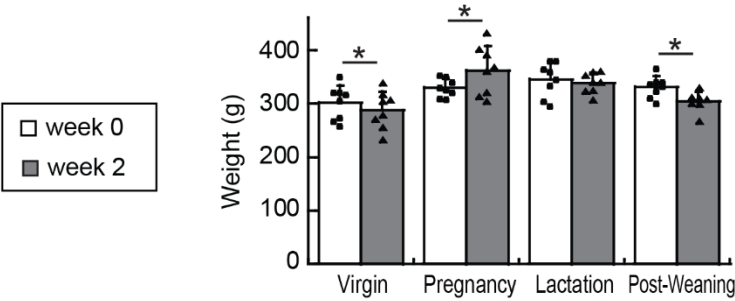
Data represented as mean ± SD.

*: Loaded different from non-loaded tibia, p<0.05.

596 **Figure S1:** Relationships between strains ($\mu\epsilon$) on the surface of the tibial midshaft and the
597 corresponding magnitude of compressive load required (N) in 4-month-old male rats, 4-month-
598 old virgin female rats, 12-month-old virgin female rats, and 12-month-old reproductive female
599 rats.

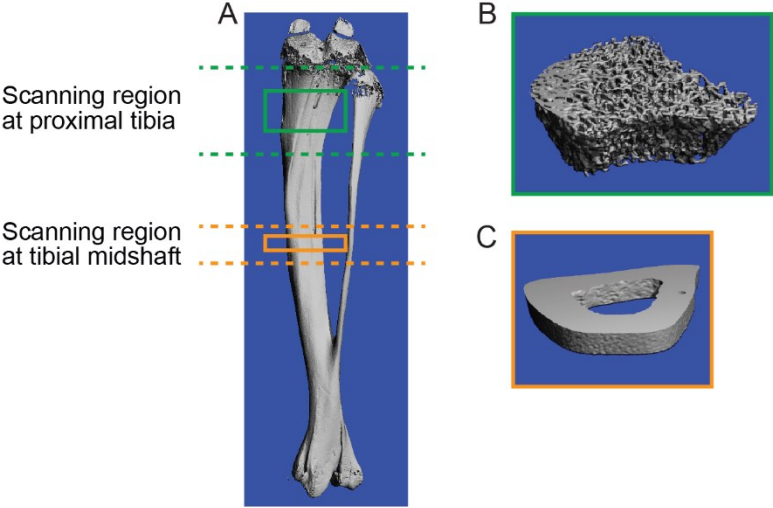


601 **Figure S2:** Weight of rats before (week 0) and after loading (week 2). * indicates significant
602 difference over the loading period ($p<0.05$).

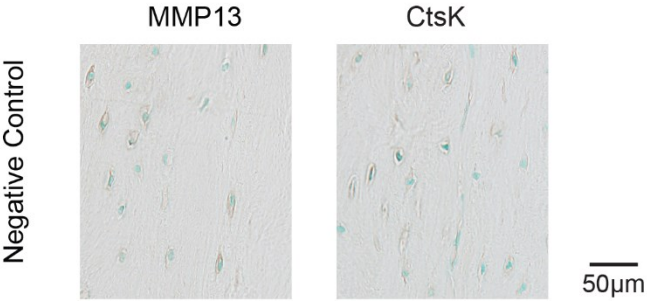


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604 **Figure S3:** (A) Illustrations of μ CT scanning regions and VOIs for analysis. Region between
605 green dash lines is the scanning region at proximal tibia; green rectangle represents the VOI for
606 trabecular bone analysis. Region between orange dash lines is the scanning region at tibial
607 midshaft; orange rectangle represents the VOI for cortical bone analysis. (B) Representative 3D
608 renderings of trabecular bone at the proximal tibia. (C) Representative 3D renderings of cortical
609 bone at the tibial midshaft.

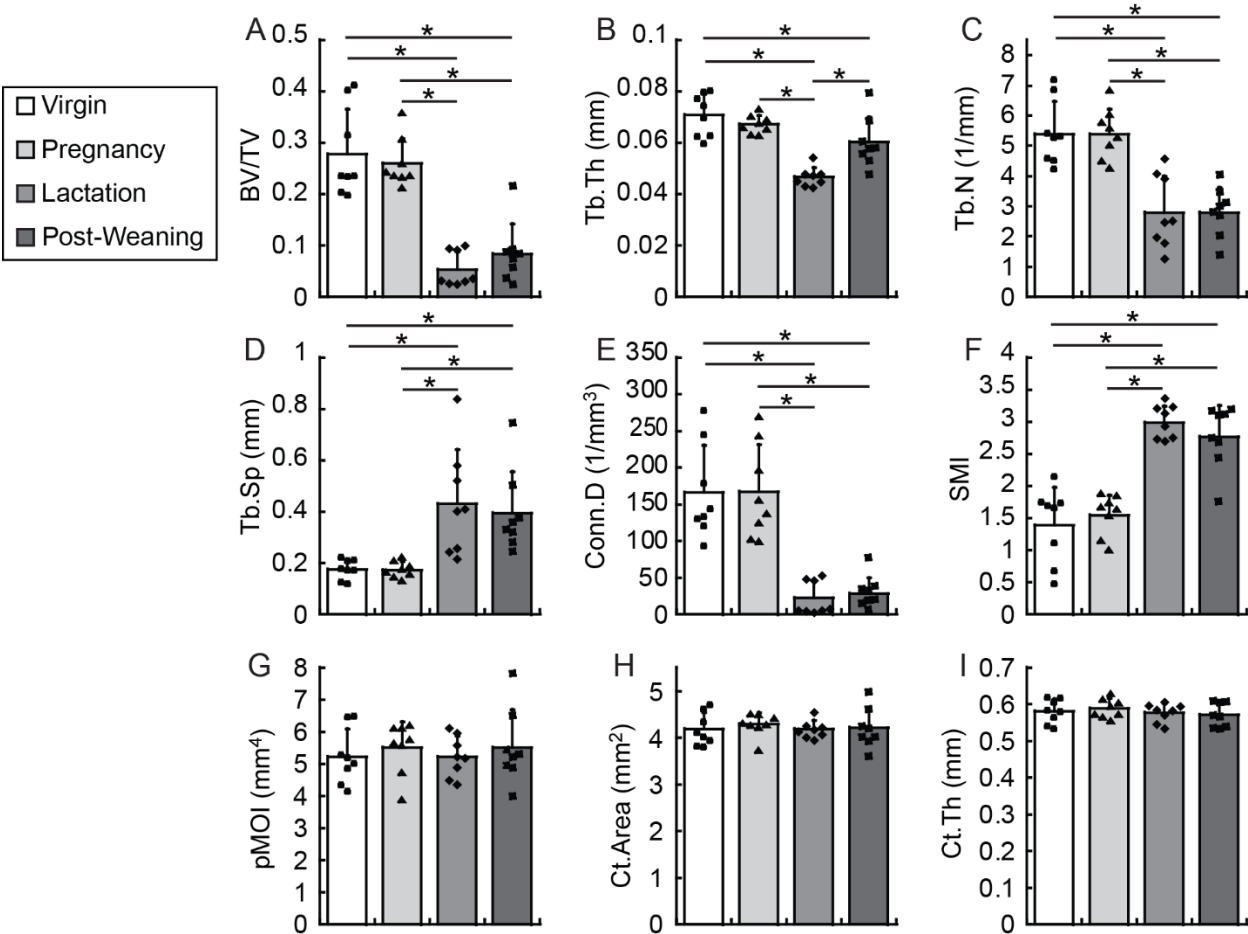


611 **Figure S4:** Representative images of negative controls of MMP13 and CtsK IHCs



612

Figure S5: Trabecular microstructural parameters at proximal tibia in rats at different reproductive stages, including: (A) BV/TV, (B) Tb.Th, (C) Tb.N, (D) Tb.Sp, (E) Conn.D, and (F) SMI. Cortical bone structural parameters at tibial midshaft in rats at different reproductive stages, including: (G) pMOI, (H) Ct.Area, and (I) Ct.Th. * indicates significant difference among groups ($p<0.05$).



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