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ORIGINAL ARTICLE

Calcium restriction during lactation has minimal effects on post-weaning mineral metabolism and bone recovery

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Abstract

Dietary calcium (Ca) restriction during lactation in the rat, which induces intra-cortical and endocortical remodeling, has been proposed as a model to study bone matrix maturation in the adult skeleton. The purpose of this study was to assess the effects of dietary Ca restriction during lactation on post-weaning mineral metabolism and bone formation. Mated female Sprague–Dawley rats were randomized into groups receiving either 0.6% Ca (lactation/normal Ca) or 0.01% Ca (lactation/low Ca) diets during lactation. Virgin animals fed normal Ca were used as controls (virgin/normal Ca). At the time of weaning, animals on the low Ca diet were returned to normal Ca and cohorts of all three groups were sacrificed at days 0, 1, 2, 7, and 14 post-weaning. Lactation caused bone loss, particularly at the endocortical surface, but the amount was not affected by dietary Ca. Rats in the lactation/low Ca group had increased cortical porosity compared to the other groups, particularly within the size range of secondary osteons. Dietary Ca restriction during lactation did not affect post-weaning bone formation kinetics or serum Ca and phosphate levels. In both lactation groups, there was a transient increase in phosphate and fibroblast growth factor 23 (FGF23) post-weaning, which trended toward virgin/normal Ca levels over time. Thus, the additional challenge of low dietary Ca during lactation to induce intra-cortical remodeling in the rat has minimal effects on bone formation kinetics and mineral metabolism during the post-weaning period, providing further justification for this model to study matrix maturation in the adult skeleton.

Keywords Lactation · Mineral Metabolism · Bone Remodeling · Calcium · Rat

Introduction

Lactation represents a challenge to the mother to provide the necessary supply of calcium (Ca) to the developing neonate [1]. The primary mechanism by which Ca is mobilized to the breast milk during lactation is through an upregulation of skeletal remodeling, which is uncoupled to favor resorption [2]. Increased skeletal resorption can be substantial, with losses of 30–40% in femoral ash weight in lactating rats [3, 4]. After weaning, bone remodeling shifts in favor of new bone formation [5, 6], leading to large regions of rapidly forming bone.

By taking advantage of the highly anabolic state following weaning, our laboratory has recently demonstrated the usefulness of the low Ca during lactation model to study matrix maturation throughout the cortical bone, including in Haversian-like remodeling units [7]. Despite the extensive bone loss due to the combination of lactation and Ca deficiency, the post-weaning bone matrix maturation process was largely unaffected by this dietary deprivation and the circulating Ca and phosphate levels were not different compared to virgin control animals fed an adequate Ca diet [7].

Adaptations in mineral metabolism associated with lactation have been described in some detail in previous reviews [1, 8, 9]. The post-weaning recovery phase, however, is not as well-characterized and to our knowledge there have been no investigations comparing the post-lactation recovery process in animals fed adequate Ca to those on a restricted Ca diet during lactation. Therefore, the current study aimed to

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investigate bone formation kinetics and mineral metabolism following weaning in rats fed normal (0.6% Ca) and low Ca (0.01% Ca) diets during lactation. Due to the anticipated large extent of bone loss associated with the combination of lactation and a low Ca diet, we hypothesized that animals fed a low Ca diet during lactation would have reduced bone formation and delayed normalization of mineral metabolism in the post-weaning recovery period.

Materials and methods

Study design

A total of 75, 12 week old female Sprague–Dawley rats were purchased from Envigo Labs. 50 of the 75 animals were bred at 10 weeks of age and shipped at E15, 6–7 days before parturition, while the remaining 25 animals were age-matched virgin controls. The animals were then individually housed in the Rush University Comparative Research Center under an institutionally approved protocol.

Upon arrival, all animals were immediately placed on a normal Ca diet (0.6% Ca, 0.4% phosphate, Teklad diets, TD.97191). Similar to our previous study [7], the experimental design included two phases: induction (lactation) and recovery (post-weaning). Induction was initiated when the animals in the timed pregnancy groups gave birth, which occurred at 13 weeks of age (±2 days). At parturition, the 50 dams were randomly assigned to (a) either the normal Ca (0.6% Ca, 0.4% phosphate) or a low Ca (0.01% Ca, 0.4% phosphate, Tekland diets, TD.9507) diet for the duration of the lactation period (i.e., the induction phase) and (b) one of 5 recovery times (days 0, 1, 2, 7 or 14 post-weaning). Virgin animals were randomly assigned to time-matched control groups. At parturition, litters were normalized to 12 pups per dam and all fostered pups were accepted without incident. On day 17 of lactation, the pups were weaned. The animals in the lactation/low Ca group were returned to the normal Ca diet. One animal in the lactation/low Ca group died during parturition and another rat in the lactation/normal Ca died 1 day after weaning, meaning that most time points had 5 animals but two had 4 animals (Table 1).

During the course of the recovery period, three animals in the lactation/normal Ca and one animal in the lactation/low Ca group developed mammary gland infections (mastitis) attributed to wounds that developed during the course of lactation. On day 2 of recovery, all animals received a prophylactic 3-day course of subcutaneous antibiotics, 100 mg/kg cefazolin. Endpoints derived from animals with mastitis were compared to non-infected animals of the same group to determine whether the localized infection impacted the study results. No outliers in any of the endpoints were noted in these animals and they were, therefore, included in the study.

Calcein (25 mg/kg, Sigma Aldrich) was injected subcutaneously to label forming bone with variable injection strategies depending on the sacrifice time. Animals sacrificed on the first day of recovery were given a single calcein injection at weaning (day 0). Animals sacrificed on day 2 were given calcein injections at days 0 and 1 of recovery. Animals sacrificed on day 7 were given calcein injections on days 3 and 6, and animals sacrificed on day 14 were given calcein injections on days 7 and 12.

At sacrifice, the left femurs were fixed in 70% ethanol. Blood was collected via cardiac puncture using a 25 G needle, allowed to clot at room temperature for ~ 30 min and centrifuged at 1000*g* for 15 min at 4 °C (Eppendorf 5415R) to separate serum.

Circulating mineral metabolism biomarkers

Ionic calcium (BioVision) and phosphate (BioVision) were assessed by colorimetric assays. Circulating mineral metabolism biomarkers were assessed using commercially available ELISA assays, including parathyroid hormone (PTH, UCSNK), parathyroid hormone related hormone (PTHrP, UCSNK), calcitonin (UCSNK), and full length fibroblast growth factor 23 (FGF23, Immuntopics). All markers were assessed at days 0, 1, 2, 7, and 14 of recovery.

Micro-computed tomography (μCT)

μCT scanning was performed using a tube voltage of 70 kVp, a current of 57 μA, an integration time of 1500 ms, and a voxel size of 2 μm (μCT50, Scanco Medical). Cortical geometry and porosity were assessed in a 500 μm thick

Table 1 Study design

Group	Induction (lactation)	Recovery (days)				
		0	1	2	7	14
Virgin/normal Ca	25	5	5	5	5	5
Lactation/normal Ca	25	5	5	4	5	5
Lactation/low Ca	25	5	5	5	4	5

The table values in the shaded cells represent the final group sizes after animal losses described in the "Study Design" section

transverse cross-sectional region of interest in the distal femoral diaphysis, located at 70% of the total femoral length. This anatomical location was previously identified as a site with significant endocortical and intra-cortical remodeling in lactation/low Ca animals [7]. Reconstructed regions were analyzed using a lower and upper threshold value of 270 and 1000, which corresponds to 617 and 3000 mg hydroxyapatite per cm^3 ($mgHA/cm^3$). Specific μCT variables measured included total area, marrow area, cortical area, and cortical porosity.

Cortical porosity was classified into three distinct variables; total cortical porosity, osteonal porosity, and lacunar porosity. Total cortical porosity is reported using conventional analysis and nomenclature [10]. The total pore volume within the size range of secondary osteons was determined by obtaining the total volume of porosity measured that had pore diameters consistent with secondary osteons, 30–60 μm, and normalizing by the total cortical bone volume analyzed. The chosen diameter range of 30–60 μm is consistent with histological characterization of the size of secondary osteons [11, 12] in rats and is a marker of intra-cortical remodeling. By definition, secondary bone is formed in place of previously resorbed bone [13]. Therefore, pores on the size of osteons were referred to as secondary osteons because in our previous publication we demonstrated that the osteons were formed in places where cortical bone had been resorbed and further, they presented with cement lines and concentric lamellae [7].

The average lacunar volume was determined by quantifying the total pore volume for pores with a diameter between 8 and 20 μm and normalizing by the total cortical bone volume analyzed. The average diameter of osteonal and lacunar porosity was determined from porosity size distributions of pore diameter vs the number of voxels of porosity within that diameter range. The average diameter was calculated as the weighted mean of the distribution gated to the diameter range of lacunae (8–20 μm) and osteons (30–60 μm).

Section preparation

After μCT scanning, femurs were dehydrated in a graded series of alcohol solutions and embedded in an epoxy resin (EpoThin, Buehler). 1 mm thick slabs at the femoral ROI were cut from the embedded specimens (Isomet 5000, Buehler) and fixed to plastic slides. The slabs were ground to a thickness of roughly 500 μm and polished to a mirror finish (Phoenix 4000, Buehler), using a series of colloidal diamond suspensions (9, 3, 1 μm average particle size, Metadi, Buehler) with ethylene glycol as a lubricant [14].

Dynamic histomorphometry

Dynamic histomorphometry was performed at the endocortical surface of the distal femoral diaphysis using fluorescent microscopy (Nikon Eclipse 80i) and commercial software (OsteoMeasure, OsteoMetrics). The primary outcome measures were the mineralizing surface per bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate per bone surface (BFR/BS). The MAR and BRR/BS parameters are dependent on double labels, and therefore it was not possible to assess these parameters in the animals sacrificed at days 0 and 1 of recovery, as these animals only received one calcein injection. MS/BS was determined using both single and double labels (MS/BS = double label + ½ single label) [15]. In cases where double labels were not clearly present, an imputed MAR value of 0.3 was assumed so that BFR/BS could be calculated.

Statistics

The study was designed with the intention of running two-way analysis of variance (ANOVA) tests to assess the effects of group (virgin/normal Ca, lactation/normal Ca, and lactation/low Ca) and time (length of time post-weaning or recovery) and to explore interactions between group and time. To ensure that the ANOVA assumptions were met, we first tested the normality of the residuals from the two-way ANOVA output using the Shapiro–Wilk test. All of the μCT variables (cortical area, medullary area, total area, and cortical porosity) were normally distributed, justifying the use of the ANOVAs, followed by post hoc comparisons using Tukey's HSD. Bone formation (MS/BS, MAR, and BFR/BS) and mineral metabolism measurements (ionic Ca, PTH, PTHrP, calcitonin, phosphate, and FGF23 concentrations) were found to be non-normally distributed. Therefore, non-parametric test were used. Group and time effects were tested separately using the Krusal–Wallis test followed by post hoc between-group comparisons using Dunn's test. Finally, non-parametric Spearman's correlation analysis was performed to test the associations between mineral metabolism marker levels and indices of bone formation.

Results

Cortical area was reduced in both lactation groups compared to the virgin/normal Ca controls at each time point during recovery (Fig. 1a). From day 0 to day 14, both lactation groups had increases in cortical area, leading to a significant overall time effect. However, there was no change in cortical area in the virgin/normal Ca controls during the recovery process, leading to a significant statistical interaction term

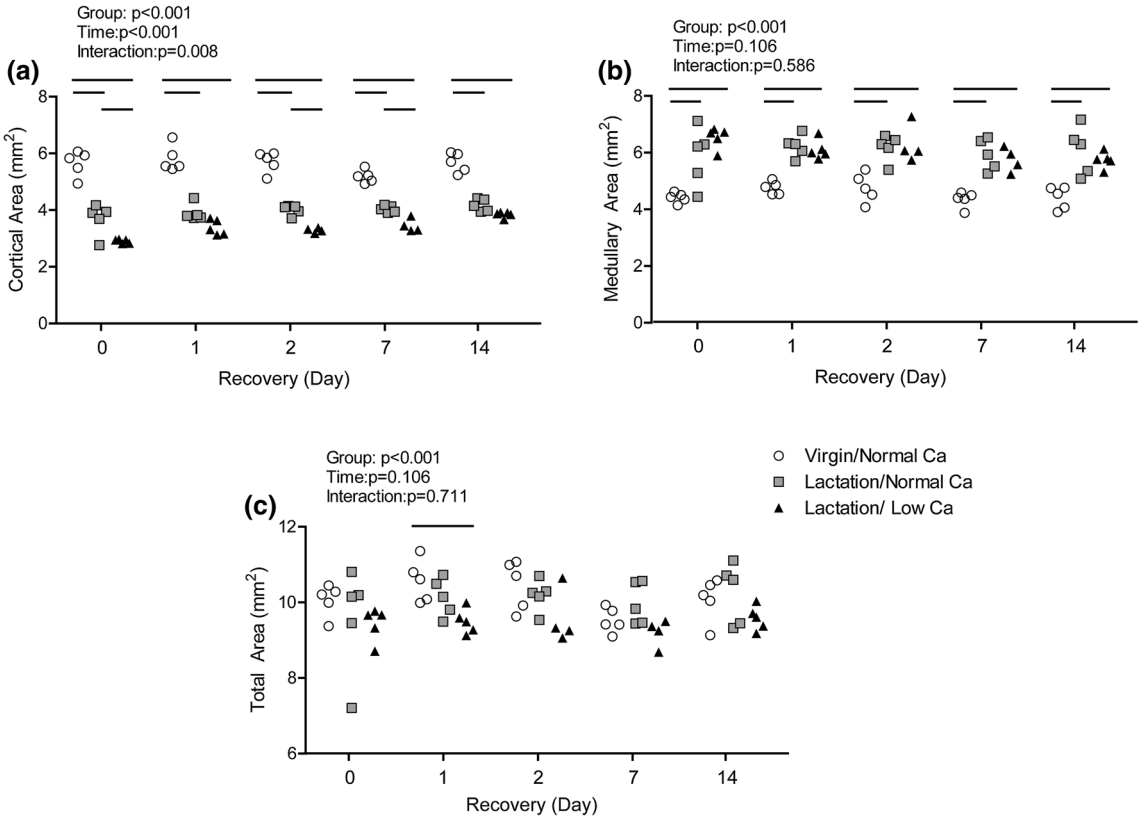

Fig. 1 µCT-derived parameters of cortical geometry measured at the distal femoral diaphysis during the post-weaning recovery period. **a** Cortical area, **b** medullary area, and **c** total area are presented for the virgin/normal Ca (open circles), lactation/normal Ca (gray square), and lactation/low Ca (black triangle) as individual animals. Results from the two-way ANOVA are presented in the legends, with between-group post hoc differences ($p < 0.05$) indicated by horizontal bars

between group and time, as the three groups demonstrated different trends over time. There were consistent between-group differences for medullary area, with significantly greater values in both lactation groups compared to the virgin/normal Ca controls at each time point (Fig. 1b). There were no significant time or interaction effects for the medullary area. Dietary Ca had less effect on total area (Fig. 1c), although the lactation/low Ca group had lower values than the virgin/normal Ca group at days 0, 1 and 2 and the lactation/normal Ca group at day 1. Similarly, there were no significant time or interaction effects for the total area.

Cortical porosity was elevated in the lactation/low Ca group compared to the other two groups at days 2 and 14, but did not change over time (Fig. 2a). The lactation/low Ca group had elevated osteonal-sized porosity compared to the lactation/normal Ca group at days 1, 7 and 14 (Fig. 2b). Osteonal porosity decreased in the lactation/low Ca group over time, but remained relatively constant in the other two groups. There was a significant time effect in the osteonal diameter with both lactation groups showing decreasing diameter over the recovery period (Fig. 2c). When the porosity data were gated to reflect lacunar-sized pores, both lactation groups had lower lacunar porosity compared to the virgin/normal controls at days 1 and 2 (Fig. 2d). By day 14, the lactation/low Ca lacunar porosity was significantly greater than the lactation/normal Ca or virgin/normal Ca groups. The average lacunar diameter was consistently greater in the lactation/low Ca group (Fig. 2e).

Bone formation indices were assessed at the endocortical surface of the distal femoral diaphysis and demonstrated a high degree of variability, necessitating the use of non-parametric tests. MAR was significantly elevated in the lactation/low Ca group compared to the virgin/normal Ca controls at day 14 (Fig. 3a). MS/BS was elevated in the lactation/low Ca group, reaching significance at day 7 (Fig. 3b). Endocortical BFR/BS was elevated in the lactation/low Ca group compared to the lactation/normal Ca at day 7 (Fig. 3c).

Metabolic biomarkers of Ca metabolism were largely unaffected by lactation or the amount of Ca in the diet (Fig. 4). Circulating ionic Ca levels varied over time in the virgin/normal Ca and the lactation/low Ca groups, with a rapid decline on day 1, followed by a stabilization of the calcium levels. The lactation/normal Ca group did not show

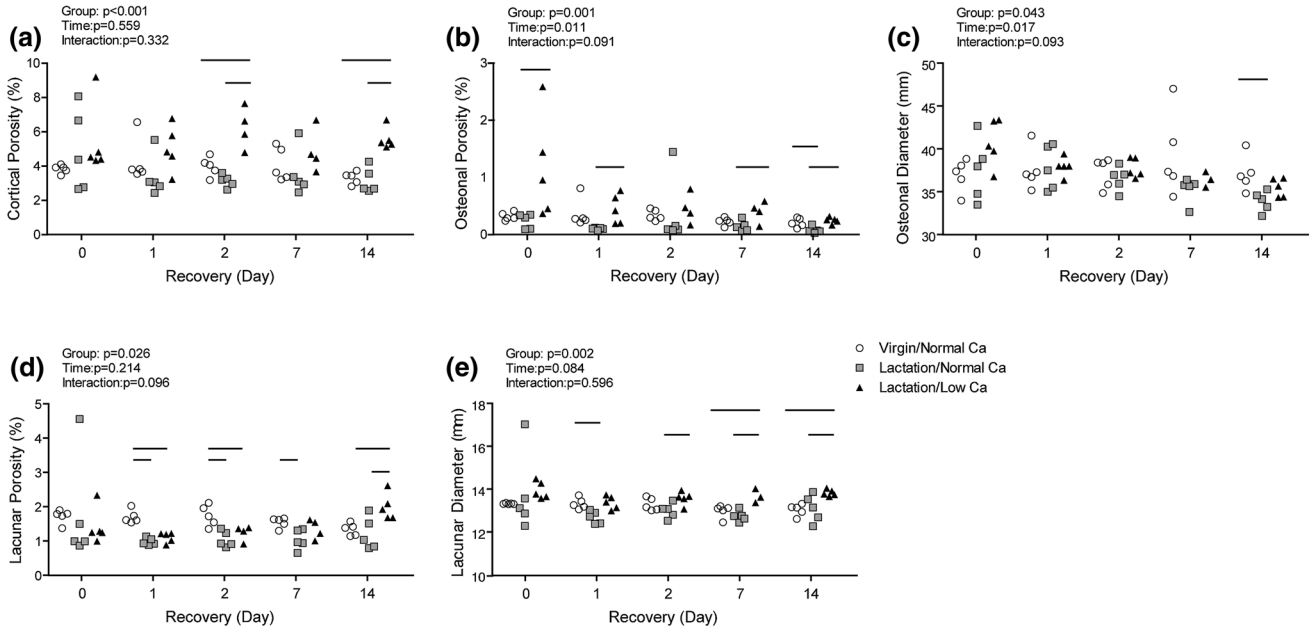

Fig. 2 µCT-derived parameters of cortical porosity measured at the distal femoral diaphysis. **a** Cortical porosity, **b** osteonal pore volume (between 30 and 60 µm diameter), and **c** lacunar pore volume (between 8 and 20 µm diameter) are presented for the virgin/normal Ca (open circles), lactation/normal Ca (gray square), and lactation/low Ca (black triangle) as individual animals. Results from the two-way ANOVA are presented in the legends, with between group post hoc differences ($p < 0.05$) indicated by horizontal bars

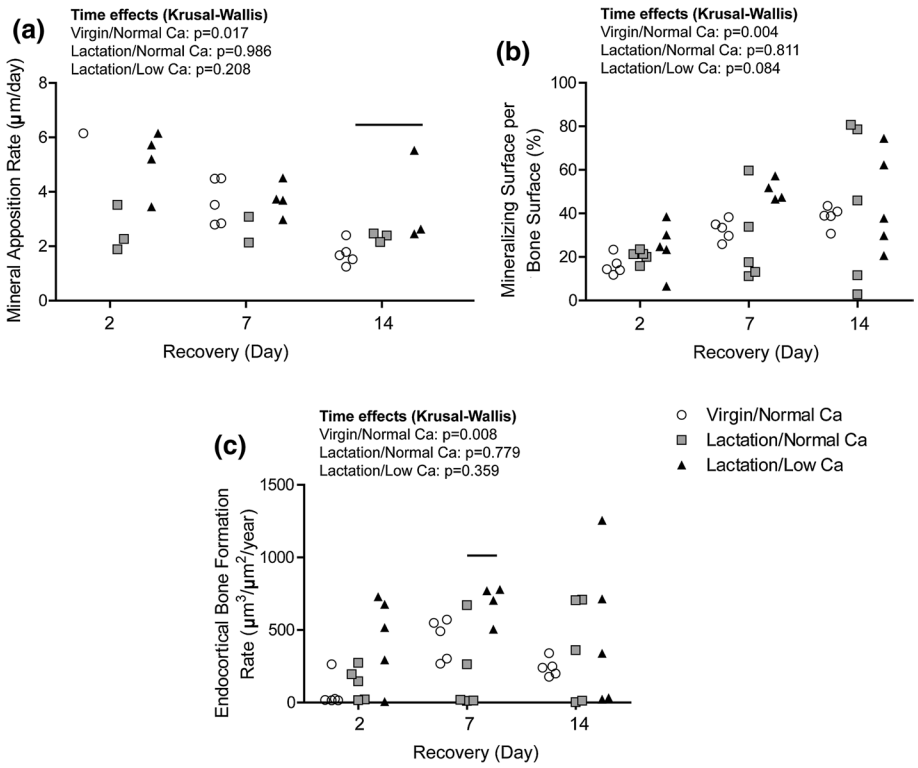

Fig. 3 Dynamic histomorphometric parameters of bone formation at the endocortical surface. The **a** bone formation rate per bone surface (BFR/BS), **b** mineral apposition rate (MAR), and **c** mineralizing surface per bone surface (MS/BS) are presented for the virgin/normal Ca (open circles), lactation/normal Ca (gray square), and lactation/low Ca (black triangle) as individual animals. Time effects for each group (Krusal–Wallis) are presented in the legends of each figure. Between group differences at each time-point were assessed using a non-parametric Mann–Whitney *U* test and significance ($p < 0.05$) are indicated with a bracket above the corresponding time-point

a significant time effect. At day 0, there were no between-group differences in the level of circulating ionic calcium, however, at day 1 Ca levels were slightly elevated in the lactation/low Ca compared to the virgin/normal Ca group. There were no between-group differences at any of the other time points measured.

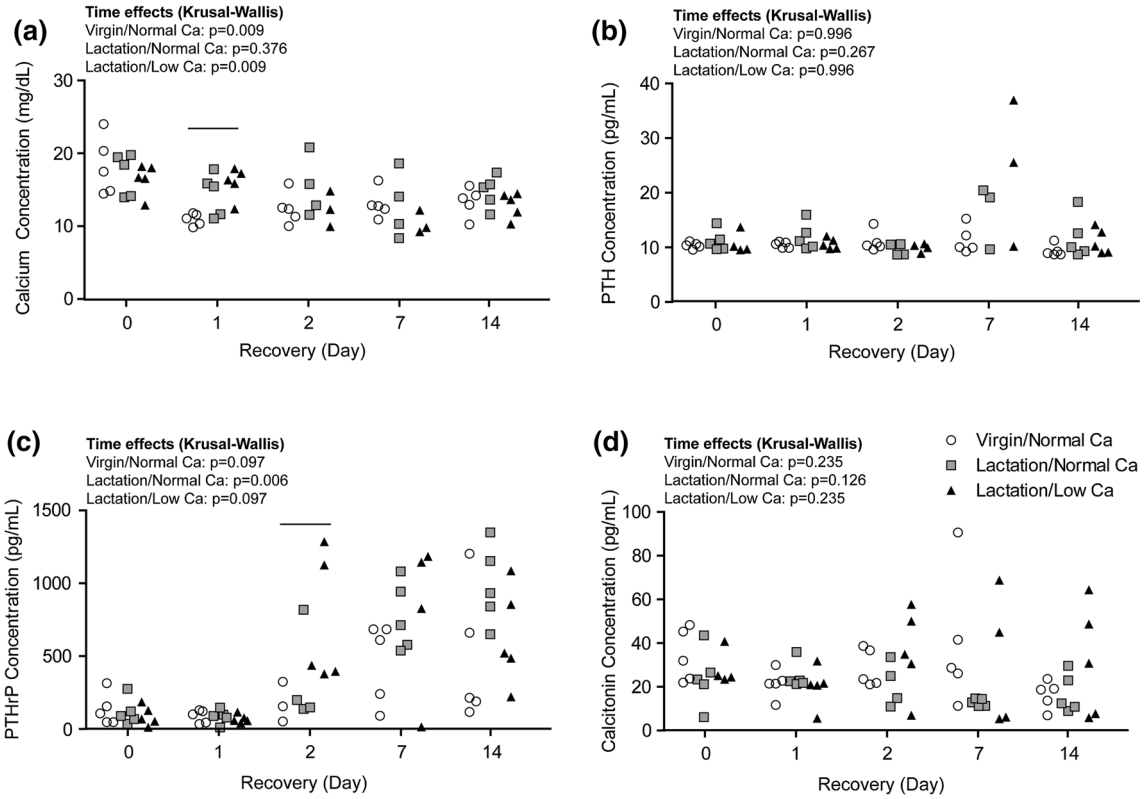

Fig. 4 Calcium metabolism measured after weaning in the recovery phase. The circulating levels of **a** ionic Ca, **b** calcitonin, **c** PTH, and **d** PTHrP are presented in the virgin/normal Ca (open circles), lactation/normal Ca (gray square), and lactation/low Ca (black triangle) as individual animals. Time effects for each group (Krusal–Wallis) are presented in the legends of each figure. Between group differences at each time-point were assessed using a non-parametric Mann–Whitney *U* test and significance ($p < 0.05$) are indicated with a bracket above the corresponding time-point

Circulating PTH levels did not vary over time for any of the groups, nor were there any between-group differences (Fig. 4b). The PTHrP levels in all three groups increased over time (Fig. 4c), however, due to high within-group variance, the only significant time effect was in the lactation/normal Ca group ($p = 0.006$). Calcitonin levels did not change over time, nor were there any between-group differences (Fig. 4d).

Circulating phosphate metabolism markers were impacted by lactation, but not by dietary Ca (Fig. 5). Phosphate varied over time in all three groups (Fig. 5a). In both lactation groups, the phosphate levels were reduced compared to the virgin/normal Ca controls at day 0, but were elevated at days 1 and 2, with no between-group differences in phosphate at days 7 and 14.

Circulating full length FGF23 levels demonstrated a high degree of within-group variability. Despite this variability, there was a significant time effect in the lactation/normal Ca group, with the other two groups failing to reach significance (Fig. 5b).The lactation/low Ca group had significantly reduced FGF23 at day 0 compared to both the virgin/normal Ca controls and the lactation/normal Ca group. However, by FGF23 levels in both lactation groups were significantly elevated compared to the virgin/normal Ca controls at days 1, 2 and 14, although the levels by day 14 were much more similar to the virgin/normal Ca control levels than at the preceding time points.

Correlations between biomarker levels and the bone formation measurements were significant for PTHrP level and both the mineralizing surface per bone surface (Spearman's rho = 0.273, p value = 0.042) and the bone formation rate (rho = 0.298, $p = 0.026$, Supplemental Table 1). Phosphate levels were negatively correlated with the mineralizing surface (rho = − 0.329, $p = 0.014$) and nearly significantly associated with the bone formation rate (rho = − 0.258, $p = 0.057$). None of the other biomarkers had significant correlations with the bone formation variables.

Discussion

To accommodate the high calcium demands of the suckling pups, lactating dams upregulate skeletal resorption leading to loss of bone mass [4, 6, 16–26]. After weaning, the dams

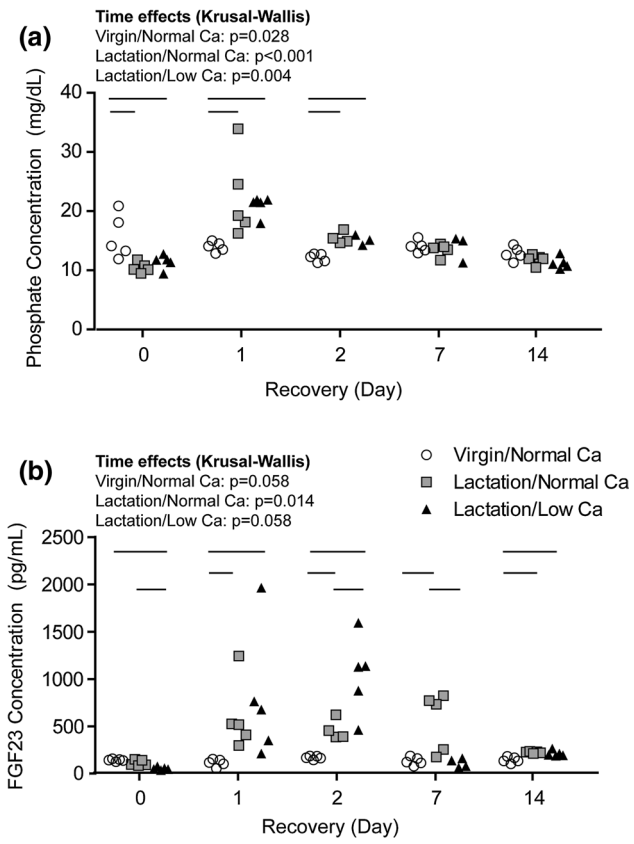

Fig. 5 Phosphate metabolism measured after weaning in the recovery phase. The circulating levels of **a** phosphate, and **b** intact FGF23 are presented in the virgin/normal Ca (open circles), lactation/normal Ca (gray square), and lactation/low Ca (black triangle) as individual animals. Time effects for each group (Krusal–Wallis) are presented in the legends of each figure. Between group differences at each time-point were assessed using a non-parametric Mann–Whitney *U* test and significance ($p < 0.05$) are indicated with a bracket above the corresponding time-point

experience a highly anabolic bone formation period wherein the lost bone mass is largely recovered [5, 21, 27, 28]. In the current study, we examined this post-weaning anabolic period by focusing on the cortical bone area and porosity, endocortical bone formation kinetics and mineral metabolism markers as a function of dietary Ca during lactation. Contrary to our hypothesis, the post-weaning bone formation kinetics and markers of mineral metabolism were largely unaffected by the amount of dietary Ca during lactation.

Similar to our previous study, at the end of lactation we found reduced cortical area in the lactation/low Ca animals, which is primarily driven by an increase in the medullary area [7]. The increased medullary area is due to elevated endocortical remodeling, which is known to occur during lactation in rats [16, 21, 29]. Surprisingly, the extent of cortical bone loss was not affected by dietary calcium during lactation, which is seemingly contrary to previously published results [30]. de Winter et al. [30] reported significantly reduced cortical bone area in lactating dams fed a low calcium diet over 7, 14, and 21 day lactation periods compared to those fed adequate calcium. It is unclear why these two studies had different findings, although it is worth noting that de Winter et al. measured cortical geometry at the midshaft, while in the current study we evaluated the distal femoral diaphysis due to our previous finding that this location is better-suited for studying both endocortical and intra-cortical remodeling [7].

Although cortical area was not significantly different between the two lactation groups, animals in the lactation/low Ca group had significantly increased cortical porosity compared to the lactation/normal Ca animals. The increase in cortical porosity can be attributed to the development of more secondary osteons due to intra-cortical remodeling based on our gating of pore size, which is consistent with our previous experiment using the lactation/low Ca model [7]. Interestingly, the porosity levels in the lactation/normal Ca group were not different from the virgin/normal Ca controls. Hagaman et al. similarly found increased cortical porosity in rats fed a low calcium diet during lactation, but no change in those fed adequate Ca during lactation [29]. Combined, these studies indicate that lactation alone primarily induces endocortical bone resorption, while the additional challenge of low dietary Ca during lactation triggers intra-cortical remodeling.

Osteocytic osteolysis is thought to play an important role in the maintenance of Ca levels during lactation [31, 32]. In the current study, the average lacunar diameter was slightly elevated in the lactation/low Ca group, which is consistent with the presence of osteocytic osteolysis. Osteocytic osteolysis is a process that has been described in mouse models of lactation [31, 33, 34], but to our knowledge there have been no published reports in the rat model.

The total fraction of porosity characteristic of lacunae was surprisingly lower in the lactation groups compared to the virgin controls. Although the current mechanism is unknown, it is possible that resorption in the lactation model targets regions of particularly high osteocyte density, leading to overall lower lacunar porosity.

Maternal mineral metabolism during pregnancy, lactation, and weaning remain a complex field of study with some conflicting findings in animal models [1, 8]. One of the mineral metabolism adaptations thought to be required to upregulate skeletal remodeling during lactation is increased production of PTH [17, 18, 24, 35–38]. Yet, our study found that PTH levels were not different between groups at the point of weaning. Despite the rapid bone formation following weaning, PTH levels have been reported to rapidly return to control levels following weaning [39, 40], which is consistent with our findings.

PTHrP levels are elevated during lactation and are likely involved in the increased skeletal remodeling [41]. The

response of PTHrP in rodents following weaning is not well-studied. Lactating women have a significant reduction in PTHrP levels 4 months after cessation of breast feeding [42]. Therefore, it is not clear how rapidly PTHrP levels respond to weaning. The current study suggests that there is an increase in PTHrP levels during the first 14 days after weaning. It is possible that PTHrP is involved in post-weaning bone accrual, as PTHrP signaling in osteoblasts has been shown to increase during bone formation [43, 44]. Indeed, circulating PTHrP levels were found to be significantly correlated with the bone formation rate and mineralizing surface per bone surface.

Circulating phosphate has previously been shown to increase following abrupt weaning [45, 46], which is consistent with the current study. Despite, the transient increase, phosphate metabolism was normalized by 14 days following weaning in the present study. The pattern of FGF23 levels in our study following weaning is consistent with the role of FGF23 in the regulation of phosphate [47, 48]. Specifically, as the phosphate levels rose during the post-weaning recovery phase, FGF23 levels increased to promote phosphate excretion in the kidney, thereby normalizing the circulating phosphate levels.

We only examined the first 14 days of the post-weaning recovery period so we could focus on the early changes in mineral metabolism and bone formation. From the μCT data, it is apparent that the rats had not fully recovered bone mass by day 14, and therefore it is possible that several of the biomarkers, such as PTHrP and FGF23, would show additional dynamic behavior in longer follow-up periods.

Although there were no obvious effects on mineral metabolism in animals that developed mastitis, we cannot fully rule out effects of this localized inflammation. Mastitis is a relatively common occurrence in lactating animals, particularly in dairy cows [49], and in the current study was likely caused by a relatively large litter size (12 pups per dam). As mastitis was only evident upon the second day post-weaning, it is also possible that the abrupt weaning strategy contributed to its development. Abrupt weaning was chosen as a method to capture the rapid transition from resorption to formation and has been used in previous studies [45, 50], but it is possible that lengthening the weaning period would have prevented mastitis development.

In the current study, we investigated the effects of dietary Ca restriction during lactation on post-weaning bone formation and mineral metabolism. Surprisingly, dietary Ca restriction during lactation did not affect the extent of cortical bone loss, although the combination of lactation and a low Ca diet induced more intra-cortical remodeling than lactation alone. Similarly, dietary calcium during lactation had no effect on post-weaning bone formation, Ca metabolism, or phosphate metabolism. Regardless of dietary calcium, lactation caused transiently elevated phosphate and FGF23 levels post-weaning, which trended toward virgin/normal Ca levels over the 14 day recovery period. We conclude that the recovery process following lactation is largely insensitive to the concentration of dietary Ca provided during lactation and provides further justification for the use of the lactation/low Ca model as a means to investigate matrix maturation during intracortical and endocortical remodeling in the adult skeleton.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Statement of animal rights All applicable institutional and/or national guidelines for the care and use of animals were followed. The articles does not contain any studies with human participants performed by any of the authors.

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