DIEGO ALEJANDRO TORRES ARBOLEDA

CHANGES IN BONE TURNOVER AND CALCIUM HOMEOSTASIS DURING REPRODUCTION IN THE BAT *Artibeus lituratus* (CHIROPTERA: PHYLLOSTOMIDAE) AND OTHER MAMMALS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Animal, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2017

DIEGO ALEJANDRO TORRES ARBOLEDA

CHANGES IN BONE TURNOVER AND CALCIUM HOMEOSTASIS DURING REPRODUCTION IN THE BAT *Artibeus lituratus* (CHIROPTERA: PHYLLOSTOMIDAE) AND OTHER MAMMALS

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Animal, para obtenção do título de *Magister Scientiae*.

ARPOVADA: 31 de março de 2017.

Fabiana Cristina Silveira Alves de Melo

Sérgio Luís Pinto da Matta

Reggiani Vilela Gonçalves
(Orientadora)

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
T693c
2017

Torres Arboleda, Diego Alejandro, 1991-
Changes in bone turnover and calcium homeostasis during reproduction in the bat A*rtibeus lituratus* (Chiroptera: Phyllostomidae) and other mammals / Diego Alejandro Torres Arboleda. – Viçosa, MG, 2017.
vii,52f. : il. (algumas color.) ; 29 cm.

Inclui anexos.
Orientador: Reggiani Vilela Gonçalves.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Morcegos. 2. Cálcio - Homeostase. 3. Lactação - Mamíferos. I. Universidade Federal de Viçosa. Departamento de Biologia Animal. Programa de Pós-graduação em Biologia Animal. II. Título.

CDD 22 ed. 599.4

"Science is a way of thinking much more than it is a body of knowledge"

-Carl Sagan-

ACKNOWLEDGMENTS

Firstly, I must express my very profound gratitude to my parents and siblings for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this dissertation. This accomplishment would not have been possible without them. Thank you, I love you infinitely.

I would like to thank my advisor Prof. Reggiani Vilela for accepting me in her laboratory, for her patience and continuous support to my research activities.

I would like to thank Prof. Gisele Lessa and the program in Animal Biology for allowing me, a foreign student, to develop my postgraduate studies. I would also like to thank Prof. Luz Elena Muñoz in Colombia for facilitating the initial contact with the Federal University of Viçosa. I am also grateful to Brazil and CAPES for funding my studies.

I would like to thank undergraduate students of the Experimental Pathology Laboratory, Vanessa Soares and Laura Vieira do Amaral, for their help in the laboratory procedures. I am also grateful to Mariáurea Maria Sarandy, PhD, for her continues help and affect.

I would like to thank Prof. Mariella B. Freitas (Lab. of Ecophysiology of bats), Prof. Jorge Dergam (Lab. of Molecular Phylogeny) and Prof. Sirlene (Lab. of Animal Morphophysiology) for allowing me to use the equipment of their laboratories. I would also like to thank Prof. Sérgio Luis Pinto da Matta (Lab. of Structural Biology) for instructing me in histological procedures and for providing me with the necessary materials.

Finally, and not because they are less important, I would like to thank my friends Estefany Ruiz, Nicole Ibagon, José Benavides, Esteban Giraldo, David Justinico and Evaristo Villalba for the long and senseless philosophical discussions, the beers, the friendship and the long nights capturing bats, thank you very much!

ABSTRACT

TORRES, Diego Alejandro, M.Sc., Universidade Federal de Viçosa, March, 2017. **Changes in bone turnover and calcium homeostasis during reproduction in the bat *Artibeus lituratus* (Chiroptera: Phyllostomidae) and other mammals**. Adviser: Reggiani Vilela Gonçalves. Co-Adviser: Mariella Bontempo Duca de Freitas.

Objective To perform a meta-analysis of studies that report changes in bone turnover and calcium homeostasis during pregnancy and lactation in mammals and study the bone changes in the frugivorous bat *Artibeus lituratus*. **Material and Methods:** We perform a search in the PUBMED and SCOPUS databases using keywords related to bone physiology and calcium homeostasis during reproduction. Using the studies found we calculated effect sizes for blood calcium concentrations, bone mineral density, bone formation markers and calciotropic hormones. To study the changes in the humerus of the bat *Artibeus lituratus* during breeding 32 females were captured. Blood and humerus were collected for histological, mechanical and electron microscopic analysis. **Results:** In total, we found 51 articles of 14 species of mammals. The concentration of calcium in the blood decreased during gestation and lactation. The bone mineral density also was decreased. Parathyroid hormone remained diminished during reproduction, similar to calcitonin. Calcitriol increased significantly during gestation. Lactating females of *A. lituratus* lost bone tissue during lactation, however, neither calcium content in bone nor mechanical parameters were different between groups. **Conclusion:** Gestation and lactation are periods of calcium stress in mammals. The mechanisms to maintain calcium homeostasis during reproduction are similar to those of non-reproductive hypocalcaemia, however, during reproduction parathyroid hormone is not involved. *A. lituratus* presents a pattern of bone loss similar to other mammals, however, seems to be minimal in this species.

RESUMO

Torres, Diego Alejandro, M.Sc., Universidade Federal de Viçosa, março de 2017. **Mudanças na remodelação óssea e homeostase do cálcio durante a reprodução no morcego *Artibeus lituratus* (Chiroptera: Phyllostomidae) e outros mamíferos**. Orientadora: Reggiani Vilela Gonçalves. Coorientadora: Mariella Bontempo Duca de Freitas.

Objetivo: Realizar uma meta-análise de estudos que relatam alterações no turnover ósseo e homeostase do cálcio durante a gravidez e lactação em mamíferos e estudar as alterações ósseas no morcego frugívoro *Artibeus lituratus*. **Material e Métodos:** Realizamos uma pesquisa nas bases de dados PUBMED e SCOPUS usando palavras-chave relacionadas à fisiologia óssea e à homeostase do cálcio durante a reprodução. Usando os estudos encontrados calculamos tamanhos do efeito para as concentrações de cálcio no sangue, densidade mineral óssea, marcadores de formação óssea e hormônios calciotrópicos. Para estudar as alterações no úmero do morcego *Artibeus lituratus* durante a reprodução 32 fêmeas foram capturadas. O sangue e o úmero foram coletados para análises histológicas, mecânica e microscopia eletrônica. **Resultados:** No total, foram encontrados 51 artigos de 14 espécies de mamíferos. A concentração de cálcio no sangue diminuiu durante a gestação e a lactação. A densidade mineral óssea também foi diminuída. A hormona paratiróide permaneceu diminuída durante a reprodução, semelhante à calcitonina. O calcitriol aumentou significativamente durante a gestação. As fêmeas lactantes de *A. lituratus* perderam tecido ósseo durante a lactação, porém, nem o conteúdo de cálcio nem os parâmetros ósseos nem mecânicos foram diferentes entre os grupos. **Conclusão:** Gestação e lactação são períodos de estresse de cálcio em mamíferos. Os mecanismos para manter a homeostase do cálcio durante a reprodução são semelhantes aos da hipocalcemia não reprodutiva, no entanto, durante a reprodução a hormona paratiróide não está envolvida. *A. lituratus* apresenta um padrão de perda óssea semelhante a outros mamíferos, no entanto, parece ser mínimo nesta espécie.

CONTENTS

Article 1: **Changes in bone turnover and calcium homeostasis during pregnancy and lactation in mammals: A meta-analysis**

ABSTRACT

Large amounts of calcium are required during pregnancy and lactation to allow fetus and newborn bone growth and calcification. An inadequate supply of calcium during these stages can lead to unsuccessfully reproduction or impaired offspring fitness. During reproduction, mammals developed different physiological changes, including adaptations to allow an adequate supply of calcium. The lack of quantitative studies that analyze these changes from a comparative perspective limits our ability to explain and understand these changes. In this work, we performed a meta-analysis of studies that report changes in bone turnover and calcium homeostasis during pregnancy and lactation in 14 species of mammals. Our meta-analysis from 51 studies showed that all species have a similar pattern of physiological changes during pregnancy and lactation, which include: (1) decreased serum calcium concentrations, (2) bone tissue loss, (3) decreased serum calcitonin and parathyroid hormone concentrations (4) increased serum calcitriol concentration, regardless of changes in parathyroid hormone concentrations. In addition, we found a negative relationship between: 1) serum calcium concentrations and the number of teats and 2) parathyroid hormone serum concentrations and litter mass.

Key words: Bone loss, bone physiology, calcium metabolism, calcium physiology, reproduction.

INTRODUCTION

Pregnancy and lactation are costly activities which increase mother's energy and nutrients demand (Speakman 2008). Not meeting these demands may lead to an unsuccessful reproduction or impaired offspring's fitness (Schmidt and Hood 2016). The specific body composition and the developmental dynamic dictate what type of nutrients are required and when they are needed (Hautier et al. 2013). In most vertebrates, a hard tissue (i.e. bone tissue) is characterized as containing large amounts of calcium and phosphates. In mammals, this tissue growth and mineralization during the last fetal phases and postnatal development implies a continuous loss of calcium from the mother's blood to the offspring through the placenta and mammary glands (Kovacs 2011; Dilworth and Sibley 2013).

In adults, blood calcium supply comes from intestinal absorption, renal reabsorption and bone tissue degradation (Olausson et al. 2012). Calcium homeostasis is highly maintained across vertebrate lineages (8-12 mg/dL) (Doherty et al. 2015) and is regulated by negative feedback systems involving the parathyroid hormone (PTH), calcitonin and calcitriol. When blood calcium concentration decreases, parathyroid cells are stimulate to produce PTH (Kantham et al. 2009), which acts on bone cells to increase bone resorption (Li et al. 2007) and in kidneys to increase calcium reabsorption (Kovacs 2005). PTH also stimulates the formation of the active form of vitamin D (i.e. calcitriol) in the kidneys. The increase of calcitriol stimulates the absorption of calcium in the intestine and inhibits the production of PTH (Christakos et al. 2011). Calcitonin, produced by parafollicular cells in the thyroid gland, does just the opposite when blood calcium concentration increases, by decreasing bone resorption by bone cells and calcium reabsorption by the kidneys (Paula

and Rosen 2010).

As the largest reserve of calcium in the body, the skeleton plays a key role on the several physiological changes occurring during pregnancy and lactation. These adaptations to allow an adequate calcium supply for fetal and postnatal development include the accumulation of bone calcium during early-mid pregnancy, the increase of calcitriol during late pregnancy and the increase of bone resorption followed by the decrease of bone mineral density (BMD) during lactation (Bowman and Miller 2001; Macica et al. 2016). Species-specific differences in reproductive characteristics influence the magnitude of these changes. For example, species with larger litter sizes (e.g. rat and mice) can deplete bone calcium reserves during lactation by up to 35% (Brommage and DeLuca 1985, Qing et al. 2012), compared to a much lower depletion (3 to 10%) of species with smaller litters (e.g. human) (Black et al. 2000, More et al. 2001).

Despite the numerous studies reporting changes on bone turnover and calcium homeostasis during pregnancy and lactation, only non-quantitative reviews have examined these changes from a comparative perspective (Bowman and Miller 2001; Wysolmerski 2002). The lack of quantitative studies, from a comparative point of view, limits generalizations and weakens our ability to better understand these changes. In order to fulfill this lack, we performed a meta-analysis of the studies that report reproductive data on blood calcium, markers of bone formation, calciotropic hormones and bone mineral density during pregnancy and lactation.

We also explored the relationship between the magnitude of these changes and the species-specific characteristics such as pregnancy and lactation length, litter size, litter mass and the number of teats. Since increasing the number of pups probably increase the

calcium demand, we expect to find a negative relationship between serum calcium and litter mass, litter size and number of teats. Long periods of gestation and lactation are also likely to subject the mother to greater calcium stress, activating mechanisms to correct calcium levels. Due to this, we expect to find a positive relationship between pregnancy and lactation length and calcitriol and PTH concentrations.

MATERIALS AND METHODS

Search strategy

We performed a literature search on the electronic databases PubMed (http://www.pubmed.gov) and Scopus (http://www.scopus.com) in August 2016 using the following keywords: “bone and bones” OR “bone remodeling” OR “bone density” OR “skeleton” AND “pregnancy” OR “lactation” OR “reproduction” AND “female”. We crossed the output of this search with a filter containing all mammalian orders and some common names (Supplemental Data S1). Only studies published in English, Portuguese and Spanish were considered. Reviews, comments, as well as unpublished studies were not included.

Study selection

The process of study selection for meta-analysis involved the following steps: (1) duplicated studies (presented in both databases) were identified and removed; (2) by examining the title and abstract, studies reporting bone and calcium physiological alterations during pregnancy and lactation were preselected; (3) by examining the sections “Material and Methods” and “Results”, preselected studies meeting the inclusion criteria

(see below) were selected; (4) we searched the references of the selected studies for other studies that met the inclusion criteria. For a study to be included, it had to meet the following criteria: (1) contemplate one or more species of mammals; (2) report reproductive (pregnancy or lactation) values of blood calcium concentration, biochemical markers of bone formation (alkaline phosphatase and osteocalcin), calciotropic hormone concentrations (PTH, calcitriol and calcitonin) and bone mineral density; (3) report the mean, sample size (n) and standard deviation (SD) or standard error (SE) of experimental groups. To compare with the other mammalian species, we searched for human studies published between 1995 and 2016 in PUBMED. This searched results in the addition to the meta-analysis of nine human studies that met the inclusion criteria.

Data extraction and analysis

We extracted from each study the values of mean, sample size (n) and the measure of variance (i.e. standard deviation or standard error). When measure of variance was reported as standard error, we transformed to standard deviation multiplying the standard error by the square root of the sample size. When data were reported in figures, we extracted the values using the software Web Plot Digitizer (Rohatgi 2016). These sample statistics were used to calculate the effect sizes, i.e. the change of a variable of a group in relation to another group, using the Hedges's g standardized mean difference (SMD) (Hedges and Olkin 1985). We used the continuous randomization method DerSimonin-Laird to calculate the cumulative effect sizes (d) for a sample of studies addressing the same effect (Dersimonian and Laird 1986; DerSimonian and Laird 2015). Confidence limits (CI) of 95% around the effect sizes were also calculated. The effect sizes were considered

significant if these limits did not overlap zero (Hedges and Olkin 1985; Lakens 2013).

The data reported in the studies are measured at different times throughout gestation and lactation. Therefore, we assigned the data to six reproductive groups: early, mid and late pregnancy and early, mid and late lactation. The distribution into these three categories was made taking into consideration the duration of pregnancy and lactation of each species. Data are presented in forest plots illustrating cumulative effect sizes comparing reproductive groups to control groups (non-reproductive females). We also compared all reproductive groups to late pregnancy group (the period in which occurs most of the calcium mobilization and the midpoint of entire reproduction period). We ran the meta-analysis using the software OpenMetaAnalyst (Wallace et al. 2012). Finally, we used simple linear regressions to test the relationship between effect sizes of calcium serum concentrations and calciotropic hormones concentrations and species-specific characteristics (body size, pregnancy and lactation length, litter size, litter mass corrected for maternal mass and number of teats). Mean values of characteristics were extracted from mammals (Jones et al. 2009) and amniote (Myhrvold et al. 2015) life-history databases. We performed regressions using the software GraphPad Prism.

RESULTS

Overview of data set

PRISMA diagram (Supplemental Data S2) illustrates the process of study selection. The initial search resulted in 3478 studies, of which 3291 were excluded because they were not related to bone turnover or calcium physiology during reproduction or were reviews or short communications; 36 were excluded because they did not report the required

reproductive parameters; and 68 were excluded because they did not report the required statistics. In total, 48 studies met the inclusion criteria. Additionally, we added three studies found in the references of the selected studies. These 51 studies, plus the 9 studies of humans, generated 644 effect sizes: 160 for calcium, 134 for osteocalcin, 82 for BALP (bone-specific alkaline phosphatase), 69 for PTH, 61 for calcitriol, 59 for alkaline phosphatase (ALP), 56 for BMD and 23 for calcitonin. The data came from 14 species of mammals from five different orders (Table 1). Ten species are domestic animals and four species are non-domestic animals maintained in experimental colonies. We did not found studies meeting the inclusion criteria that report bone and calcium changes during reproduction in mammals captured in the wild.

Calcium

Serum calcium concentration decreased significantly during mid (spp. = 4; d = -0.695; CI = -1.202/-0.188) and late pregnancy (spp. = 7; d = -0.808; CI = -1.022/-0.594) in most species (Fig. 1A). During early lactation, serum calcium also decreased significantly in most species except in the crab-eating macaque and human (spp. = 5; d = -0.596, CI = -0.972/-0.22). Serum calcium during early (spp. = 10; d = 0.282, CI = 0.05/0.515) and mid lactation (spp. = 8; d = 0.747, CI = 0.305/1.19) were higher compared to late pregnancy (Fig 1B), except the pig that showed a decrease during early lactation (n = 8; d = -0.317, CI = -0.717/ 0.083).

Bone Mineral Density

Effect sizes related to BMD during pregnancy and lactation were available only for human,

rat and guinea pig. BMD showed a significant decrease in BMD during all pregnancy in the three species (spp. = 3; d = -0.444, CI = -0.684/-0.204) (Fig 2A). BMD at lumbar vertebrae and femur tend to decrease during pregnancy, but this decrease was only significant at the tibia (spp. = 2; d = -0.846, CI = -1.592/-0.099) (Fig 2B). BMD decrease was also significant during all lactation (spp. = 2; d = -1.044, CI = -1.364/-0.724) (Fig 2A). Femur, tibia and lumbar vertebrae showed a significant decrease during all lactation (Fig. 2B).

Calciotropic hormones

PTH decreases significantly during all pregnancy (spp. = 5; d = -0.426, CI = -0.75/-0.101) and during all lactation also tended to decrease but not significantly (spp. = 5; d = -0.216, CI = -0.547/0.114) (Fig.3A). Compared to late pregnancy, PTH was significantly lower at mid-pregnancy (spp. = 3; d = -0.537, CI = -0.973/-0.101) and significantly higher at early lactation (spp. = 8; d = 0.645, CI = 0.06/1.231) (Fig. 3B). Calcitonin decreases significantly during all pregnancy (spp. = 2; d = -0.42, CI = -0.828/-0.012) and during all lactation tended to decrease but not significantly (spp. = 2; d = -0.118, CI = -0.711/0.475) (Fig. 4A). Calcitonin is higher during all lactation in relation to late pregnancy (spp. = 5; d = 0.524, CI = 0.128/0.92) (Fig. 4B).

Calcitriol increases significantly during mid (spp. = 2; d = 2.036, CI = 0.657/3.414) and late pregnancy (spp. = 6; d = 2.149, CI = 0.732/3.566) in all species (Fig. 5A). At early and mid-pregnancy calcitriol was lower than during late pregnancy (spp. = 5; d = -1.7, CI = -2.49/-0.91) (Fig. 5B). Calcitriol during all lactation tended to increase but not significantly (spp. = 5; d = 1.634, CI = -0.007/3.274) and it tended to be lower during all lactation (spp. = 6; d = -0.382, CI = -1.18/0.42) than during late pregnancy (Fig. 5B), except the goat that

showed higher calcitriol concentration during early (n = 4; d = 1.685, CI = 0.53/2.84) and mid lactation (n = 2; d = 2.893, CI = 2.083/3.702).

Bone formation markers

Osteocalcin decreases significantly during pregnancy in all species (spp. = 7; d = −1.109, CI = −1.576/−0.642) (Fig. 6A). Osteocalcin showed not significant change during all lactation (d = 0.069, CI = -0.448/0.587), except for the pig that decreased (n = 2; d = -1.165, CI = -1.692/-0.637) during early lactation. During early and mid pregnancy osteocalcin tended to be greater than during late pregnancy in all species, except the human than significantly decreased during mid pregnancy (n = 4; d = -0.727, CI = -1.097/-0.358). During early lactation, osteocalcin also is higher than during lactation in most species (spp. = 7; d = 0.333, CI = 0.045/0.62) (Fig. 6B) and only the pig showed lower concentrations (n = 2; d = -0.734, CI = -1.254/-0.214).

ALP tend to increase during all pregnancy but it was not significant (d = 0.454, CI = -0.07/0.978) (Fig. 7A). During lactation serum ALP increases significantly (d = 1.14, CI = 0.26/ 2.02) (Fig. 7A). In relation to late pregnancy, ALP was significantly lower at early and mid-pregnancy (d = -1.526, CI = -2.185/-0.866) and higher during all lactation (d = 1.078, CI = 0.252/1.904) (Fig. 7B). The bone specific ALP (BALP) do not change during all pregnancy (d = -0.015, CI = -0.892/0.862) and all lactation (d = -0.43, CI = -1.402/0.542). BALP is significantly lower at all lactation than during late pregnancy (d = -0.526, CI = -0.942/-0.109) (Fig. 8B).

The serum calcium during lactation was negatively related to the number of teats (r2 0.12; p 0.028) (Fig. 9). Only PTH were negatively related to litter mass during lactation (r2

0.41; p 0.032) (Fig. 9). Pregnancy and lactation lengths were not related to the changes observed in serum calcium, calcitriol or PTH (Fig. 10).

DISCUSION

Despite the large differences in both size and food habits, all species analyzed showed a similar pattern of changes on bone turnover and calcium homeostasis during pregnancy and lactation: (1) decreased serum calcium from mid pregnancy to early lactation; (2) bone loss throughout reproduction; (3) increased calcitriol during mid and late pregnancy and (4) decreased PTH and calcitonin concentrations during pregnancy. In addition, we found that the reduction of serum calcium during lactation was higher in species with large number of nipples such as rats and mice. We also found that the reduction in serum PTH during lactation was higher in species with higher litter body mass.

Bone turnover and calcium homeostasis regulation during reproduction

The results of the meta-analysis support the idea that pregnancy and lactation are periods of calcium stress for mammals (Kovacs 2005; 2011). We found that calcium concentrations were low during the period from mid-gestation to early lactation. This decrease was accompanied by two largely recognized changes: an increase of calcitriol concentration (Christakos et al. 2011) and bone resorption (Bowman and Miller 2001).

Calcitriol is known for increasing serum calcium concentration by promoting the absorption of dietary calcium in the intestine, increasing calcium reabsorption in the kidneys and stimulating osteoclasts for bone resorption (Kovacs 2012). We found a one-fold increase of calcitriol during mid and late pregnancy; however, the concentration of

serum calcium during mid and late pregnancy remained lower, suggesting that this mechanism is not enough to reestablish calcium homeostasis. This increase on calcitriol concentration also might account for the reduction of BMD observed at pregnancy.

During non-reproductive hypocalcaemia the production of calcitriol in the kidneys is PTH-dependent. However, we found that PTH remained low throughout all pregnancy. This implies that other hormones might increase calcitriol levels during this period (Kirby et al. 2013). One of the alternative mechanisms proposed for explaining this increase involves the parathyroid hormone-related protein (PTHrP), which is produced in the placenta, fetus tissues and uterus, and its production increases as the fetus grows (Wysolmerski 2012). As a result, low PTH concentrations observed during pregnancy are probably due to high calcitriol concentrations, since calcitriol is known to inhibit PTH synthesis and secretion on parathyroid cells (DeLuca 2004).

Similar to PTH, we found low calcitonin concentrations during reproduction. Calcitonin is a hormone that reduces calcium concentration in blood mainly through the inhibition of calcium liberation from bone (Davey and Findlay 2013). We also found this hormone higher during early lactation than late pregnancy, supporting the view that calcitonin plays a key role during lactation because it protects the skeleton from excessive bone resorption (Woodrow et al. 2006) by inhibiting osteocytic osteolysis (Clarke et al. 2015).

Bone resorption, performed by osteoclasts, is another mechanism that increase the concentration of serum calcium (Peacock 2010). We found a decrease in BMD, which indicates bone resorption, in three species analyzed and this confirms the pattern found in many other species of mammals that also lost bone tissue during pregnancy and lactation

(e.g. white-footed mice, bats and dogs) (Kwiecinski et al. 1987, Bernard and Davison 1996, Vajda et al. 1999, Schmidt and Hood 2014). We found that bone resorption was more severe in lactation than pregnancy. It is largely known that high PTHrP and prolactin concentrations during lactation are associated with bone resorption during lactation. (Charoenphandhu et al. 2010; Wysolmerski 2012). In addition, during lactation, low estrogen concentration may also exacerbate the loss of bone (Vanhouten and Wysolmerski 2003).

One of the most used bone formation marker is ALP (Seibel 2005), an enzyme produced in the intestine, placenta, liver, kidneys and bone (Golub and Boesze-Battaglia 2007). In this study, we found ALP concentrations to be lower during early and mid-pregnancy than during late pregnancy, possibly due to the contribution of the growing placenta (Kovacs 2005). At lactation, ALP increased significantly compared to non-reproductive levels, indicating a possible increase in bone formation during this period. However, as we found that BALP did not change during lactation and pregnancy, and this is a specific ALP produced by osteoblasts, it is probably that bone formation is not increased during reproduction.

Osteocalcin is another bone formation marker, secreted by osteoblasts during bone formation process (Zoch et al. 2016). The decrease of this protein that we found during pregnancy has been attributed to an increased uptake of osteocalcin by the placenta (Tabatabaei et al. 2014). However, Farrugia et al. (1989) found no evidence of this placental effect in sheep. It is possible that osteocalcin production do not decrease during pregnancy, but seems to be reduced due to the increase of renal clearance (Naylor et al. 2000). The pattern that we found in this bone formation marker vary considerably between species; for

example, osteocalcin decreased at lactation in pig, sheep and cow and at the same time increased in rat, mice, human and monkeys. Interspecific variation in renal clearance might account for these differences in osteocalcin and the other bone formation markers.

Species-specific characteristics and bone turnover

Mammary glands are the main target of calcium lost during lactation and generally, the number of teats are related to the number of pups. The greater the number of pups fed, the greater the loss of calcium through the glands (Speakman 2008; Hood 2012). This view is supported by the negative relationship between total serum calcium and the number of teats that we found during lactation. It is important to note that although we do not find support for a negative relationship between serum calcium concentration during lactation and litter size, an experimental study varying the litter size in guinea pigs has proportionated evidence for this relation (Symonds et al. 1978).

We conclude that reproduction is a period of calcium stress for mammals, which is more severe in species with heavier litters. Females respond to the demand of calcium increasing the calcitriol concentrations and bone resorption, although these mechanisms do not seem to completely meet the calcium demands because its concentration remain low during mid pregnancy until early lactation. The mechanisms of calcium regulation during reproduction are similar to those of normal hypocalcaemia, except for PTH role on calcium mobilization. Finally, the changes of bone formation markers vary greatly between species and do not show a clearly relation with patterns of bone loss.

ACKNOWLEDGMENTS

This work is part of the dissertation master of D. A. Torres, who was funded by the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) of Brazil.

REFERENCES

Bernard RTF, Davison A. 1996. Does calcium constrain reproductive activity in insectivorous bats? Some empirical evidence for Schreibers' long-fingered bat (Miniopterus schreibersii). South African Journal of Zoology 31:218–220.

Black AJ, Topping J, Durham B, Farquharson RJ, Fraser WD. 2000. A detailed assessment of alterations in bone turnover, calcium homeostasis, and bone density in normal pregnancy. Journal Bone Mineral Research 15:557–563.

Bowman BM, Miller SC. 2001. Skeletal adaptations during mammalian reproduction. Journal of musculoskeletal and neuronal interactions 1:347–355.

Brommage R, Deluca HF. 1985. Regulation of bone mineral loss during lactation. The American journal of physiology 248:182-187.

Charoenphandhu N, Wongdee K, Krishnamra N. 2010. Is prolactin the cardinal calciotropic maternal hormone? Trends in endocrinology and metabolism: 21:395–401.

Christakos S, Dhawan P, Porta A, Mady LJ, Seth T. 2011. Vitamin D and intestinal calcium absorption. Molecular and Cellular Endocrinology 347:25–29.

Clarke MV, Russell PK, Findlay DM, Sastra S, Anderson PH, Skinner JP, Atkins GJ, Zajac JD, Davey RA. 2015. A role for the calcitonin receptor to limit bone loss during lactation in female mice by inhibiting osteocytic osteolysis. Endocrinology 156:3203–3214.

Davey RA, Findlay DM. 2013. Calcitonin: physiology or fantasy? Journal of Bone and Mineral Research 28:973-979.

Deluca HF. 2004. Overview of general physiologic features and functions of vitamin D. The American journal of clinical nutrition 80:1689–1696.

Dersimonian R, Laird N. 1986. Meta-Analysis in Clinical Trials. Statistics in medicine 188:177–188.

Dersimonian R, Laird N. 2015. Meta-analysis in clinical trials revisited. Contemporary Clinical Trials 45:139–145.

Dilworth MR, Sibley CP. 2013. Review: Transport across the placenta of mice and women. Placenta 34:S34–S39.

Doherty AH, Ghalambor CK, Donahue SW. 2015. Evolutionary physiology of bone: bone metabolism in changing environments. Physiology 30:17–29.

Farrugia, W, Fortune CL, Heath J, Caple IW, Wark JD. 1989. Osteocalcin as an index of osteoblast function during and after ovine pregnancy. Endocrinology 125:1705–1710.

Golub EE, Boesze-Battaglia, K. 2007. The role of alkaline phosphatase in mineralization. Current Opinion in Orthopaedics 18:444–448.

Hautier L, Bennett, NC, Viljoen H,, Howard L, Milinkovitch MC, Tzika AC, Goswami A, Asher RJ. 2013. Patterns of ossification in southern versus northern placental mammals. Evolution 67:1994–2010.

Hedges LV, Olkin I. 1985. Statistical methods for meta-analysis. Academic Press, New York.

Kantham L, Quinn SJ, Egbuna OI, Baxi K, Butters R, Pang JL, Pollak MR, Goltzman D, Brown EM. 2009. The calcium-sensing receptor (CaSR) defends against hypercalcemia independently of its regulation of parathyroid hormone secretion. American journal of physiology. Endocrinology and metabolism 297:915-23.

Kovacs CS. 2005. Calcium and bone metabolism during pregnancy and lactation. Journal of Mammary Gland Biology and Neoplasia 10:105–118.

Kovacs CS. 2011. Calcium and bone metabolism disorders during pregnancy and lactation. Endocrinology and Metabolism Clinics of North America 40:795–826.

Kovacs, CS. 2012. The Role of Vitamin D in pregnancy and lactation: insights from animal models and clinical studies. Annual Review of Nutrition 32:97–123.

Kovacs CS. 2016. Maternal mineral and bone metabolism during pregnancy, lactation and post-weaning recovery. Physiological Reviews 96:449-547.

Kwiecinski GG, Krook L, Wimsatt WA. 1987. Annual skeletal changes in the little brown bat, Myotis lucifugus lucifugus, with particular reference to pregnancy and lactation. The American journal of anatomy 178:410–420.

Lakens D. 2013. Calculating and reporting effect sizes to facilitate cumulative science: A practical primer for t-tests and ANOVAs. Frontiers in Psychology 4:1–12.

Li X, QIN L, Bergenstock M, Bevelock LM, Novack DV, Partridge NC. 2007. Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. Journal of Biological Chemistry 282:33098–33106.

Liesegang A, Risteli J, Wanner M. 2007. Bone metabolism of milk goats and sheep during second pregnancy and lactation in comparison to first lactation. Journal of Animal Physiology and Animal Nutrition 91:217–225.

Macica CM, King HE, Wang M, Mceachon CL, Skinner CW, Tommasini SM. 2016. Novel anatomic adaptation of cortical bone to meet increased mineral demands of reproduction. Bone 85:59–69.

More C, Bettembuk P, Bhattoa HP, Balogh A. 2001. The effects of pregnancy and lactation

on bone mineral density. Osteoporosis International 12:732–737.

Naylor KE, Iqbal P, Fledelius C, Fraser RB, Eastell R. 2000. The effect of pregnancy on bone density and bone turnover. Journal of bone and mineral research 15:129–137.

Olausson H, Goldberg GR, Laskey MA, Schoenmakers I, Jarjou LMA, Prentice A. 2012. Calcium economy in human pregnancy and lactation. Nutrition Research Reviews 25:40–67.

Paula FJ, Rosen CJ. 2010. Back to the future: Revisiting parathyroid hormone and calcitonin control of bone remodeling. Hormone and Metabolic Research 42:299–306.

Qing H, Ardeshirpour L, Pajevic PD, Dusevich V, Jahn K, Kato S, Wysolmerski J, Bonewald L. 2012. Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. Journal of Bone and Mineral Research 27:1018–1029.

Schmidt CM, Hood W. 2014. Bone loss is a physiological cost of reproduction in white-footed mice (Peromyscus leucopus). Mammalian Biology 79:96–100.

Schmidt CM, Hood W. 2016. Female White-Footed Mice (Peromyscus leucopus) trade off offspring skeletal quality for self-maintenance when dietary calcium intake is low. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 325:581-587.

Speakman JR. 2008. The physiological costs of reproduction in small mammals. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 363:375–398.

Suntornsaratoon P, Krishnamra N, Charoenphandhu N. 2015. Positive long-term outcomes from presuckling calcium supplementation in lactating rats and the offspring. American journal of physiology. Endocrinology and metabolism 308:1010-1022.

Symonds HW, Bubar RH, Crackel W, Twardock AR. 1978. The effect of litter size on

placental blood flow and placental calcium transfer in multifoetate guinea-pig. British Journal of Nutrition 39:347-365.

Tabatabaei N, Rodd CJ, Kremer R, Khavandgar Z, Murshed M, Weiler HA. 2014. Dietary vitamin D during pregnancy has dose-dependent effects on long bone density and architecture in guinea pig offspring but not the Sows. Journal of Nutrition 25:1985–1994.

Taylor TG, Lewis PE, Balderstone O. 1975. Role of Calcitonin in protecting the skeleton during pregnancy and lactation. Journal of Endocrinology 66:297–298.

Vajda EG, Kneissel M, Muggenburg B, Miller SC. Increased intracortical bone remodeling during lactation in Beagle Dogs. 1999. Biology of Reproduction 61:1439–1444.

Wallace BC, Dahabreh IJ, Trikalinos TA, Lau J, Trow P, Schmid CH. 2012. Closing the gap between methodologists and end-users: R as a computational back-end. Journal of Statistical Software 49:1–15.

Woodrow JP, Sharpe CJ, Fudge NJ, Hoff AO, Gagel RF, Kovacs C. 2006. Calcitonin plays a critical role in regulating skeletal mineral metabolism during lactation. Endocrinology 147:4010–4021.

Wysolmerski, JJ. 2012. Parathyroid hormone-related protein: An Update. The Journal of Clinical Endocrinology and Metabolism 97:2947–2956.

Zoch ML, Clemens TL, Riddle RC. 2016. New insights into the biology of osteocalcin. Bone 82:42–49.

TABLES AND FIGURES

Table 1. Species of mammals included in the meta-analysis.

SUPERORDINAL GROUP	ORDER	SPECIES	COMMON NAME
Laurasiatheria	Cetartiodactyla	*Bubalus bubalis*	Buffalo
		Bos taurus	Cow
		Capra aegagrus hircus	Goat
		Ovis aries	Sheep
		Odocoileus virginianus	White-tailed deer
	Carnivora	*Canis lupus familiaris*	Dog
	Perissodactyla	*Equus caballus*	Horse
		Sus scrofa	Pig
Euarchontoglires	Primates	*Macaca fascicularis*	Crab-eating macaque
		Homo sapiens	Human
		Macaca nemestrina	Southern pig-tailed macaque
	Rodentia	*Cavia porcellus*	Guinea Pig
		Mus musculus	Mice
		Rattus norvergicus	Rat

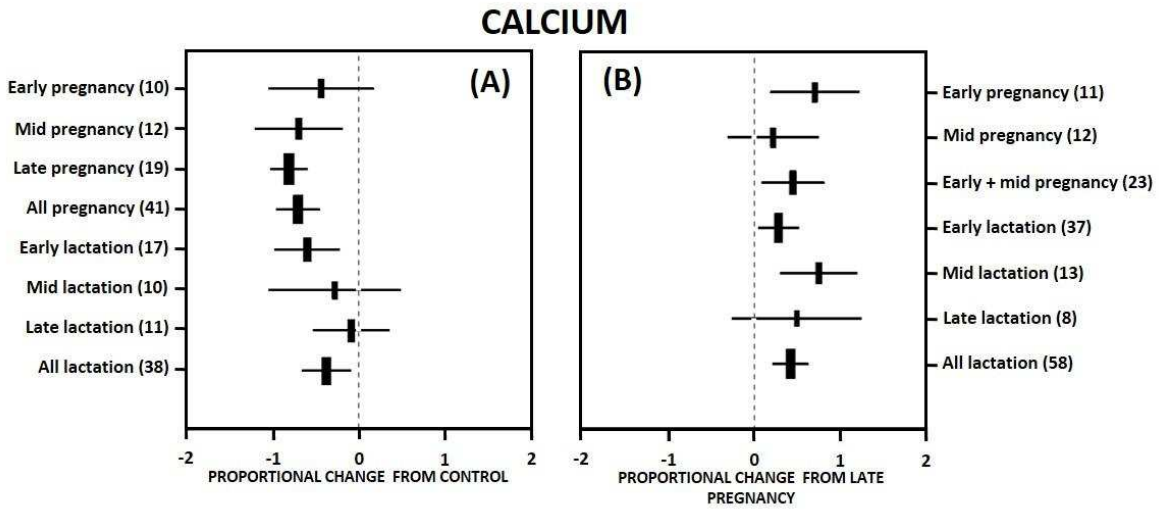


Figure 1. Blood calcium (Ca) of pregnant and lactating females in relation to non-reproductive females (A) or females in late pregnancy (B). The effect sizes are reported with their 95% confidence intervals. Effects are significant if the confidence intervals do not overlap zero. Numbers in parentheses indicate the number of independent comparisons for each effect.

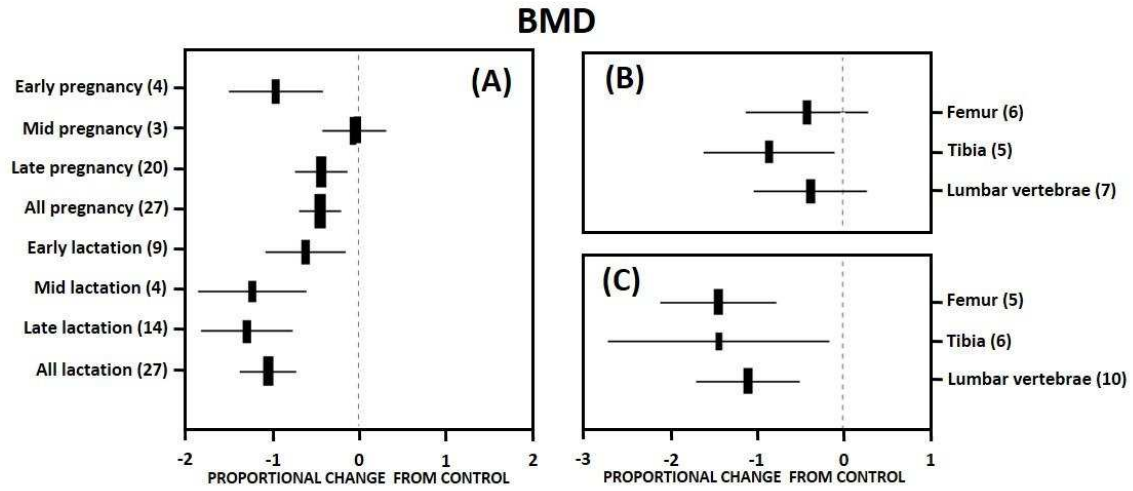


Figure 2. Bone mineral density (BMD) of pregnant and lactating females in relation to non-reproductive females (A). BMD of femur, tibia and lumbar vertebrae during pregnancy (B) and lactation (C) in relation to non-reproductive females. The effect sizes are reported with their 95% confidence intervals. Effects are significant if the confidence intervals do not overlap zero. Numbers in parentheses indicate the number of independent comparisons for each effect.

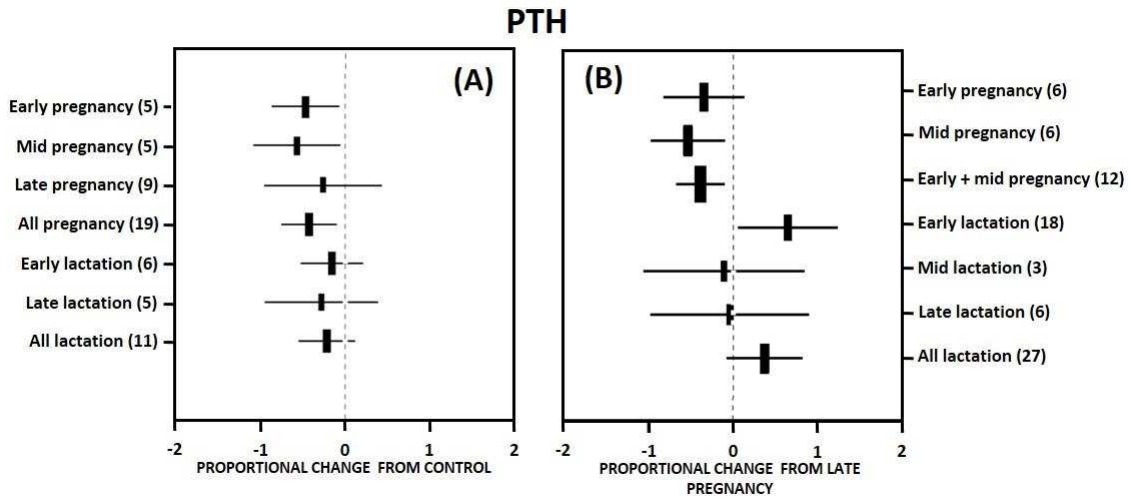


Figure 3. Parathyroid hormone (PTH) of pregnant and lactating females in relation to non-reproductive females (A) or females in late pregnancy (B). The effect sizes are reported with their 95% confidence intervals. Effects are significant if the confidence intervals do not overlap zero. Numbers in parentheses indicate the number of independent comparisons for each effect.

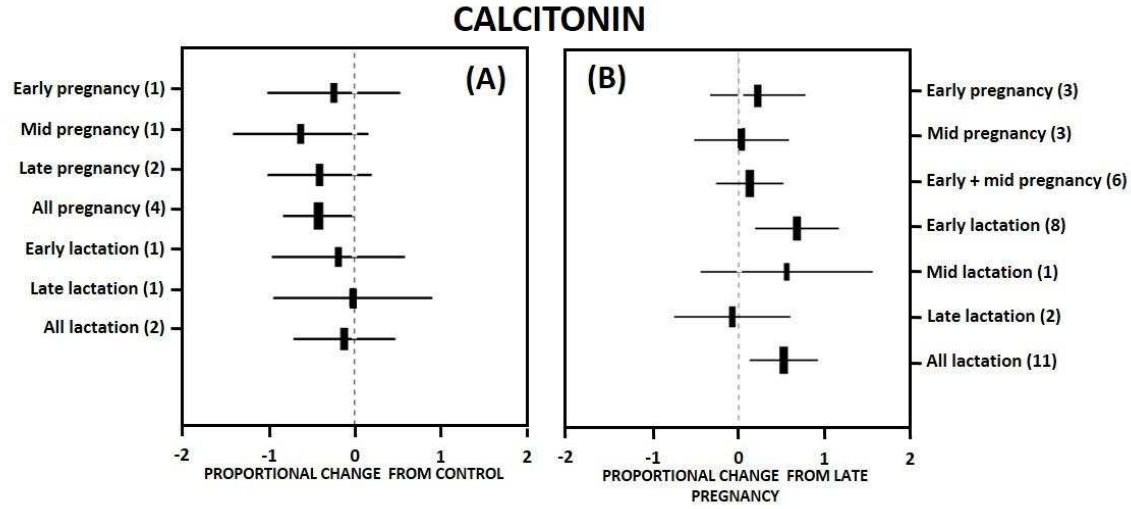


Figure 4. Calcitonin of pregnant and lactating females in relation to non-reproductive females (A) or females in late pregnancy (B). The effect sizes are reported with their 95% confidence intervals. Effects are significant if the confidence intervals do not overlap zero. Numbers in parentheses indicate the number of independent comparisons for each effect.

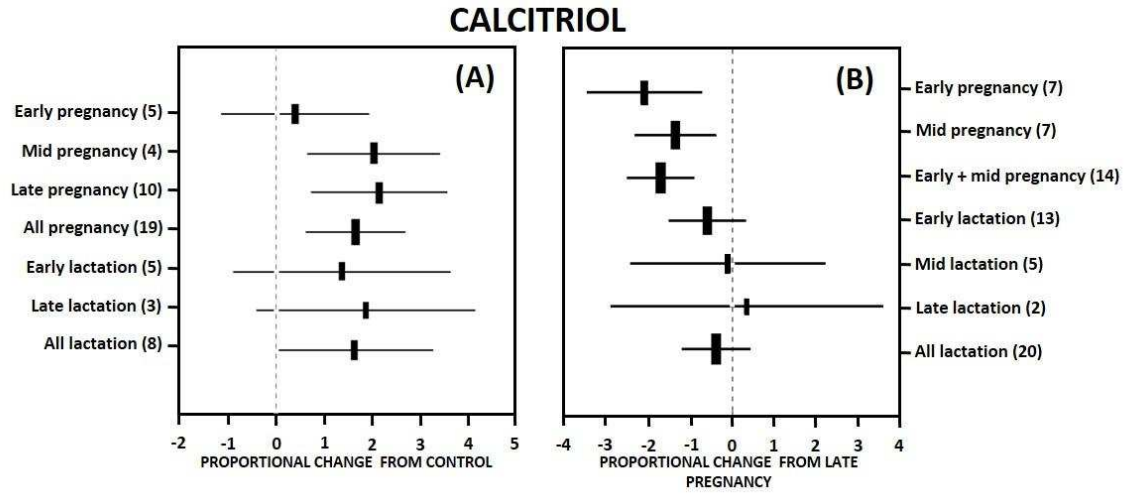


Figure 5. Calcitriol of pregnant and lactating females in relation to non-reproductive females (A) or females in late pregnancy (B). The effect sizes are reported with their 95% confidence intervals. Effects are significant if the confidence intervals do not overlap zero. Numbers in parentheses indicate the number of independent comparisons for each effect.

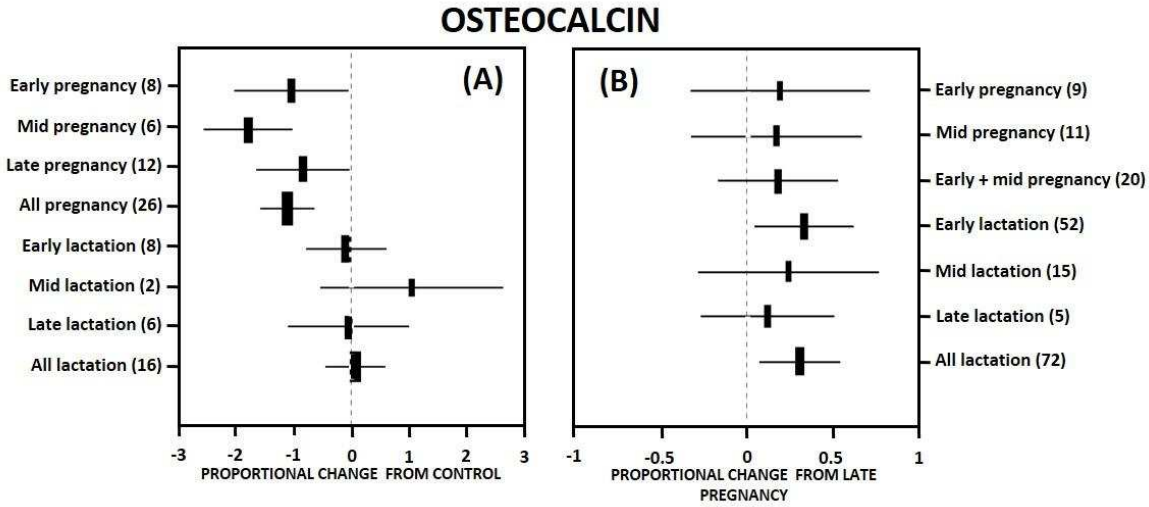


Figure 6. Osteocalcin of pregnant and lactating females in relation to non-reproductive females (A) or females in late pregnancy (B). The effect sizes are reported with their 95% confidence intervals. Effects are significant if the confidence intervals do not overlap zero. Numbers in parentheses indicate the number of independent comparisons for each effect.

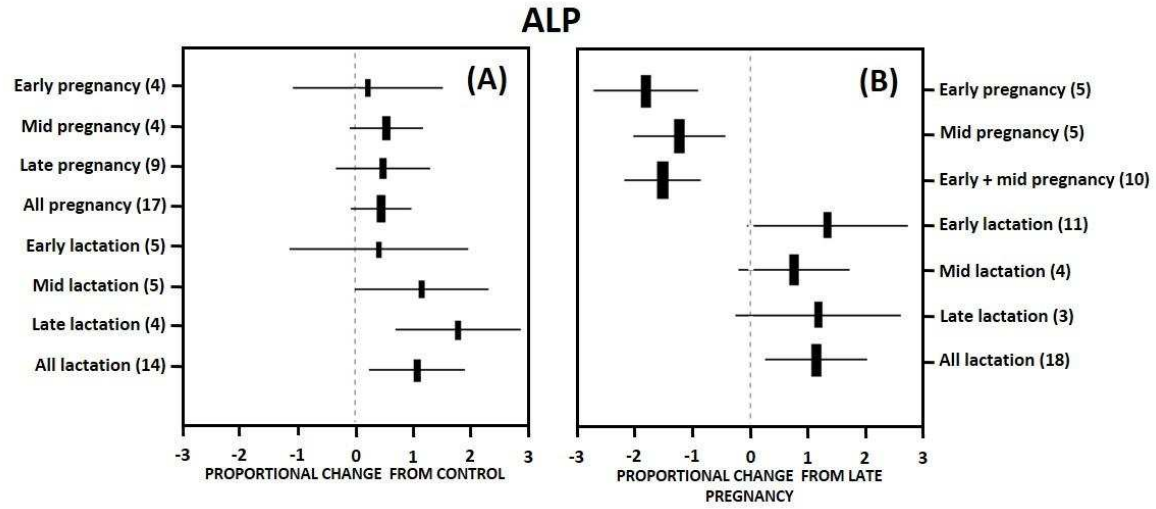


Figure 7. Alkaline phosphatase (ALP) of pregnant and lactating females in relation to non-reproductive females (A) or females in late pregnancy (B). The effect sizes are reported with their 95% confidence intervals. Effects are significant if the confidence intervals do not overlap zero. Numbers in parentheses indicate the number of independent comparisons for each effect.

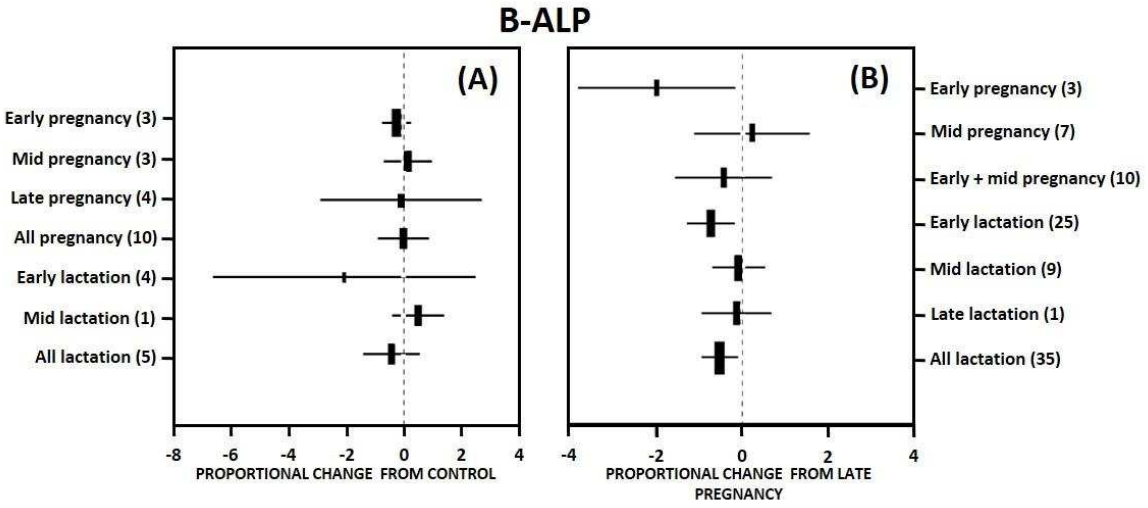


Figure 8. Bone-specific alkaline phosphatase (BALP) of pregnant and lactating females in relation to non-reproductive females (A) or females in late pregnancy (B). The effect sizes are reported with their 95% confidence intervals. Effects are significant if the confidence intervals do not overlap zero. Numbers in parentheses indicate the number of independent comparisons for each effect.

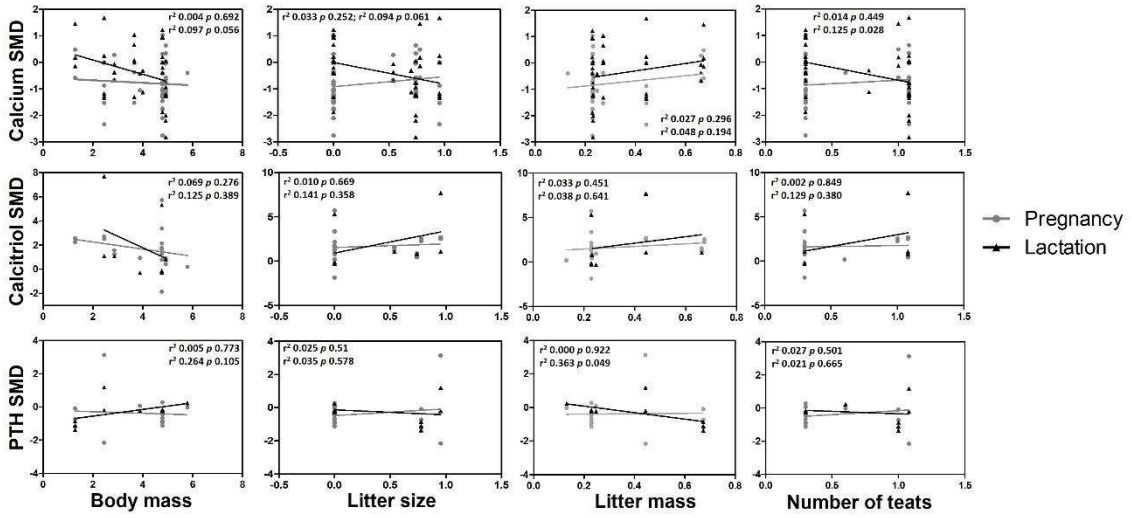


Figure 9. Relationship of the effect sizes of calcium, calcitriol and PTH during pregnancy and lactation with species-specific characteristics. Body mass, litter size and number of teats are log transformed. Litter mass is arsine transformed.

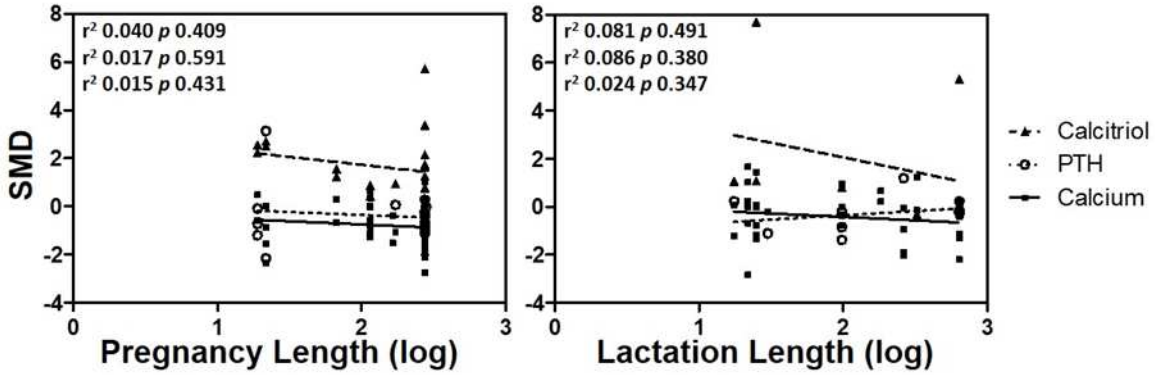


Figure 10. Relationship of the effect sizes of calcium, calcitriol and PTH with during pregnancy and lactation lengths. Lengths are log transformed.

SUPPLEMENTAL DATA S1. Search keywords.

KEYWORD USED IN PUBMED

#1 ("bone remodeling"[MeSH Terms] OR "bone and bones"[MeSH Terms] OR "bone density"[MeSH Terms] OR "Skeleton"[MeSH Terms] OR "bone remodeling"[TIAB] OR "bone and bones"[TIAB] OR "bone density"[TIAB] OR "Skeleton"[TIAB] OR "Bone turnover"[TIAB] OR "Bone Mineral content"[TIAB] OR "Bone Loss"[TIAB])

#2 ("Reproduction"[MeSH Terms] OR "Lactation"[MeSH Terms] OR "Pregnancy"[MeSH Terms] OR "Reproduction"[TIAB] OR "Lactation"[TIAB] OR "Pregnancy"[TIAB])

#3 ("female"[MeSH Terms] OR "female"[TIAB])

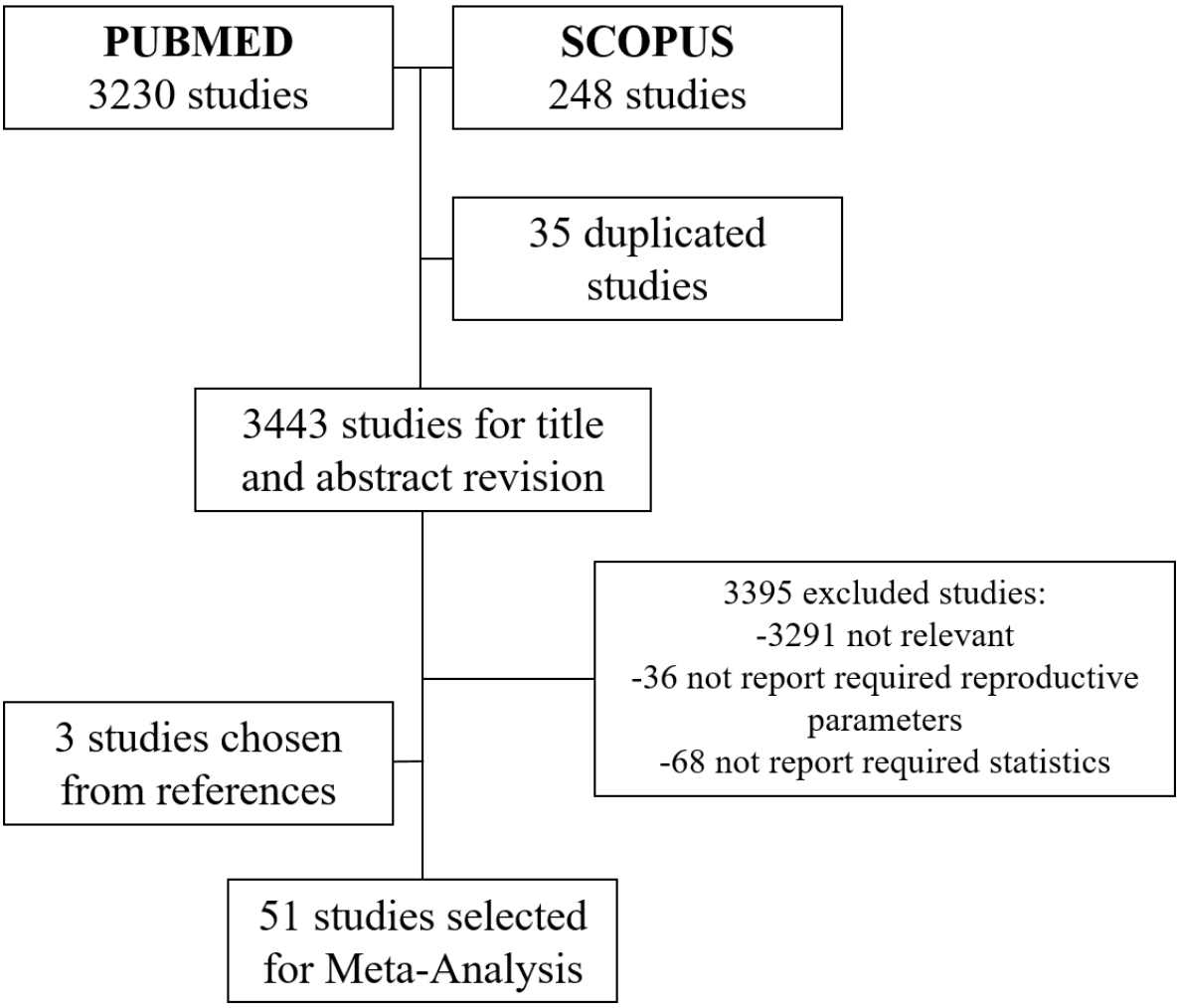
#4 ("mammals"[MeSH Terms] OR "opossums"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "dogs"[MeSH Terms] OR "cats"[MeSH Terms] OR "cattle"[MeSH Terms] OR "livestock"[MeSH Terms] OR "goats"[MeSH Terms] OR "sheep, domestic"[MeSH Terms] OR "horses"[MeSH Terms] OR "ruminants"[MeSH Terms] OR "shrews"[MeSH Terms] OR "arvicolinae"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "trichechus"[MeSH Terms] OR "proboscidea mammal"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "sloths"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "armadillos"[MeSH Terms] OR "tupaiidae"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "lemur"[MeSH Terms] OR "primates"[MeSH Terms] OR "hominidae"[MeSH Terms] OR "pongo"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "gorilla gorilla"[MeSH Terms] OR "haplorhini"[MeSH Terms] OR "hares"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "rabbits"[MeSH Terms] OR "rodentia"[MeSH Terms OR "mice"[MeSH Terms] OR "rats"[MeSH Terms] OR "moles"[MeSH Terms] OR "hedgehogs"[MeSH Terms] OR "whales"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "dolphins"[MeSH Terms] OR "artiodactyla"[MeSH Terms] OR "swine"[MeSH Terms] OR "camels"[MeSH Terms] OR "giraffes"[MeSH Terms] OR "deer"[MeSH Terms] OR "antelopes"[MeSH Terms] OR "equidae"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "ursidae"[MeSH Terms] OR "procyonidae"[MeSH Terms] OR "raccoons"[MeSH Terms] OR "mephitidae"[MeSH Terms] OR "mustelidae"[MeSH Terms] OR "pinnipedia"[MeSH Terms] OR "strepsirhini"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "cricetinae"[MeSH Terms] OR "pig"[TIAB] OR "bat"[TIAB] OR "monkey"[TIAB] OR "bitch"[TIAB] OR "hamster"[TIAB])

KEYWORDS USED IN SCOPUS

(TITLE-ABS-KEY("Reproduction") OR TITLE-ABS-KEY("Lactation") OR TITLE-ABS-KEY("Pregnancy")) AND (TITLE-ABS-KEY("bone remodeling") OR TITLE-ABS-KEY("bone") OR TITLE-ABS-KEY("bone density") OR TITLE-ABS-KEY("Skeleton"))

AND (TITLE-ABS-KEY(“mammals”))

SUPPLEMENTAL DATA S2. Preferred Reporting Items for *Systematic Reviews* and Meta-Analyses (PRISMA) illustrating the process of study selection.



SUPPLEMENTAL DATA S3. Sixty **s**tudies selected for meta-analysis: 51 studies from literature search and 9 women studies chosen by authors.

1. Ardawi MSM, Nasrat HA, BA'Aqueel HS. 1997. Calcium-regulating hormones and parathyroid hormone-related peptide in normal human pregnancy and postpartum: a longitudinal study. European journal of endocrinology 137:402–409.
2. Ardeshirpour L, Dann P, Adams DJ, Nelson T, VanHouten J, Horowitz MC, Wysolmerski JJ. 2007. Weaning Triggers a Decrease in Receptor Activator of Nuclear Factor-κB Ligand Expression, Widespread Osteoclast Apoptosis, and Rapid Recovery of Bone Mass after Lactation in Mice. Endocrinology 148:3875–3886.
3. Black AJ, Topping J, Durham B, Farquharson RG, Fraser WD. 2000. A detailed assessment of alterations in bone turnover, calcium homeostasis, and bone density in normal pregnancy. Journal of Bone Mineral Research 15:557–563.
4. Bowman BM, Siska CC, Miller SC. 2002. Greatly increased cancellous bone formation with rapid improvements in bone structure in the rat maternal skeleton after lactation. Journal of Bone Mineral Research 17:1954–1960.
5. Brommage R, Baxter DC. 1990. Inhibition of bone mineral loss during lactation by Cl2MBP. Calcified Tissue International 47:169–172.
6. Chao CC, Brown RD, Deftos LJ. 1985. Metabolism of calcium and phosphorus during pregnancy and lactation in white-tailed deer. Acta Endocrinologica 109:269–275.
7. Ciaramella P, Piantedosi D, De Luna R, Oliva G, Consalvo F, Persechino A. 2000. Biochemical indicators of bone metabolic activity in buffalo (Bubalus bubalis) during late pregnancy and early lactation. Journal of veterinary medicine. A, Physiology, pathology, clinical medicine 47:431–437.
8. Clarke MV, Russell PK, Findlay DM, Sastra S, Anderson PH, Skinner JP, Atkins GJ, Zajac JD, Davey RA. 2015. A role for the calcitonin receptor to limit bone loss during lactation in female mice by inhibiting osteocytic osteolysis. Endocrinology 156:3203–3214.
9. Cross NA, Hillman LS, Allen SH, Krause GF, Vieira NE. 1995. Calcium homeostasis and bone metabolism during pregnancy, lactation, and postweaning: a longitudinal study. The American journal of clinical nutrition 61:514–523.
10. Davicco MJ, Faulconnier Y, Coxam V, Dubroeucq H, Martin-Rosset W, Barlet JP. 1994. Systemic bone growth factors in light breed mares and their foals. Archives internationales de physiologie, de biochimie et de biophysique 102:115–119.
11. Espino L, Guerrero F, Suarez ML, Santamarina G, Goicoa A, Fidalgo LE. 2003. Long-term effects of dietary anion-cation balance on acid-base status and bone morphology in reproducing ewes. Journal of Veterinary Medicine Series A: Physiology Pathology Clinical Medicine 50:488–495.
12. Farrugia W, Fortune CL, Heath J, Caple IW, Wark JD. 1989. Osteocalcin as an index of osteoblast function during and after ovine pregnancy. Endocrinology 125:1705–1710.
13. Filipović N, Stojević Z, Prvanović N, Tuček Z. 2014. The influence of late pregnancy and lactation on bone metabolism in mares. Research in Veterinary Science 97:194–198.
14. Filipovic N, Stojevic Z, Zdelar-Tuk M, Kusec V. 2008. Plasma parathyroid hormone-related peptide and bone metabolism in periparturient dairy cows. Acta veterinaria

Hungarica 56:235–244.
15. Fudge NJ, Kovacs C. 2010. Pregnancy up-regulates intestinal calcium absorption and skeletal mineralization independently of the vitamin D receptor. Endocrinology 151:886–895.
16. Fukuda S, Lida H. 1993. Histomorphometric changes in iliac trabecular bone during pregnancy and lactation in Beagle dogs. Journal of Veterinary Medical Science 55:569.
17. Girard CL, Robert S, Matte JJ, Farmer C, Martineau GP. 1996. Serum concentrations of micronutrients, packed cell volume, and blood hemoglobin during the first two gestations and lactations of sows. Canadian Journal of Veterinary Research 60:179–185.
18. Gonen E, Sahin I, Ozbeck M, Kovalak E, Yologlu S, Ates Y. 2005. Effects of Pregnancy and lactation on bone mineral density and their relation to the serum calcium, phosphorus, calcitonin and parathyroid hormone levels in rats. Journal of Endocrinological Investigation 4:322–326.
19. Gruber HE, Stover SJ. 1994. Maternal and weanling bone: the influence of lowered calcium intake and maternal dietary history. Bone 15:167–176.
20. Honda A, Kurabayashi T, Yahata T, Tomita M, Matsushita H, Takakuwa K, Tanaka K. 2000. Effects of pregnancy and lactation on trabecular bone and marrow adipocytes in rats. Calcified Tissue International 67:367–372.
21. Ichikawa F, Katagiri K, Higuchi Y, Takeda S, Saito K. 2000. 1α-Hydroxyvitamin D 3 prevents the decrease of bone mineral density in lactating Beagles. Journal of Veterinary Medical Science 6.
22. Kirby BJ, Ma Y, Martin HM, Favaro, KLB, Karaplis AC, Kovacs CS. 2011. Skeletal recovery after weaning does not require PTHrP. Journal of Bone and Mineral Research 26:1242–1251.
23. Kirby BJ, Ma Y, Martin HM, Favaro KLB, Karaplis AC, Kovacs CS. 2013. Upregulation of calcitriol during pregnancy and skeletal recovery after lactation do not require parathyroid hormone. Journal of Bone and Mineral Research 28:1987–2000.
24. Lappeteläinen R, Lappeteläinen E, Hassinen T, Hahl M. 1993. Biochemical indicators of bone metabilc activity in bovine periparturient hypocalcemia. Journal of Veterinary Medicine 40:67–72.
25. Lees CJ, Jerome CP. 1998. Effects of pregnancy and lactation on bone in cynomolgus macaques: Histomorphometric analysis of iliac biopsies. Bone 22:545–549.
26. Lees CJ, Jerome CP, Register TC, Carlson CS. 1998. Changes in bone mass and bone biomarkers of cynomolgus monkeys during pregnancy and lactation. Journal of Clinical Endocrinology and Metabolism 83:4298–4302.
27. Liesegang A., Loch L, Bürgi E, Risteli J. 2005. Influence of phytase added to a vegetarian diet on bone metabolism in pregnant and lactating sows. Journal of Animal Physiology and Animal Nutrition 89:120–128.
28. Liesegang A, Risteli J, Wanner M. 2006. The effects of first gestation and lactation on bone metabolism in dairy goats and milk sheep. Bone 38:794–802.
29. Liesegang A, Risteli J, Wanner M. 2007. Bone metabolism of milk goats and sheep during second pregnancy and lactation in comparison to first lactation. Journal of Animal Physiology and Animal Nutrition 91:217–225.
30. Liesegang, A. 2008. Influence of anionic salts on bone metabolism in periparturient

dairy goats and sheep. Journal of dairy science 91:2449–2460.
31. Liu XS, Ardeshirpour L, Vanhouten JN, Shane E. Wysolmerski JJ. 2012. Site-specific changes in bone microarchitecture, mineralization, and stiffness during lactation and after weaning in mice. Journal of Bone and Mineral Research 27:865–875.
32. Lozupone E, Favia A. 1988. Distribution of resorption processes in the compacta and spongiosa of bones from lactating rats fed a low-calcium diet. Bone 9:215–224.
33. Miller MA, Omura TH, Miller SC. 1989. Increased cancellous bone remodeling during lactation in beagles. Bone 10:279–85.
34. Miller SC, Bowman BM. 2004. Rapid improvements in cortical bone dynamics and structure after lactation in established breeder rats. The anatomical record. Part A, Discoveries in molecular, cellular, and evolutionary biology 276:143–149.
35. Miller S, Shupe J, Redd E, Miller M, Omura T. 1986. Changes in bone mineral and bone formation rates during pregnancy and lactation in rats. Bone 7:283–287.
36. More C, Bettembuk P, Bhattoa HP, Balogh A. 2001. The effects of pregnancy and lactation on bone mineral density. Osteoporosis International 12:732–737.
37. More C, Bhattoa HP, Bettembuk P, Balogh A. 2003. The effects of pregnancy and lactation on hormonal status and biochemical markers of bone turnover. European Journal of Obstetrics Gynecology and Reproductive Biology 106:209–213.
38. Nishiwaki M, Yasumizu T, Hoshi K, Ushijima H. 1999. Effect of pregnancy, lactation and weaning on bone mineral density in rats as determined by dual-energy X-ray absorptiometry. Endocrine Journal 46:711–716.
39. Ott SM, Lipkin EW, Newell-Morris L. 1999. Bone physiology during pregnancy and lactation in young macaques. Journal of Bone and Mineral Research 14:1779–1788.
40. Paoletti AM, Orrù M, Floris L, Guerriero S, Ajossa S, Romagnino S, Melis GB. 2003. Pattern of bone markers during pregnancy and their changes after delivery. Hormone Research 59:21–29.
41. Purdie D, Aaron J, Selby P. 1988. Bone histology and mineral homeostasis in human pregnancy. British Journal of Obstetrics and Gynaecology 95:849–54.
42. Qing H, Ardeshirpour L, Pajevic PD, Dusevich V, Jahn K, Kato S, Wysolmerski J, Bonewald L. 2012. Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. Journal of Bone and Mineral Research 27:1018–1029.
43. Radojkovic D, Pesic M, Ristic T. 2014. Bone turnover markers in medicamentous and physiological hyperprolactinemia in female rats. Vojnosanitetski pregled 71:559–564.
44. Ritchie LD, Fung EB, Halloran BP, Turnlund JR, Van Loan MD, Cann CE, King JC. 1998. A longitudinal study of calcium homeostasis during human pregnancy and lactation and after resumption of menses. The American Journal of Clinical Nutrition 67:693–701.
45. Rummens K, van Bree R, Van Herck, Zaman Z, Bouillon R, Van Assche FA, Verhaeghe J. 2002. Vitamin D deficiency in guinea pigs: Exacerbation of bone phenotype during pregnancy and disturbed fetal mineralization, with recovery by 1,25(OH)2D3 infusion or dietary calcium-phosphate supplementation. Calcified Tissue International 71:364–375.
46. Sengupta S, Arshad M, Sharma S, Dubey M, Singh MM. 2005. Attainment of peak bone mass and bone turnover rate in relation to estrous cycle, pregnancy and lactation in colony-bred Sprague-Dawley rats: Suitability for studies on pathophysiology of bone and therapeutic measures for its management. Journal of Steroid Biochemistry and Molecular

Biology 94:421–429.
47. Shahtaheri SM, Aaron JE, Johnson DR, Paxton SK. 1999. The impact of mammalian reproduction on cancellous bone architecture. Journal of anatomy 194:407–21.
48. Shankar K, HidestrandM, Haley R, Skinner, RA, Hogue W, Jo C, Simpson P, Lumpkin CK, Aronson J, Badger TM, Ronis MJJ. 2006. Different molecular mechanisms underlie ethanol-induced bone loss in cycling and pregnant rats. Endocrinology 147:166–178.
49. Tabatabaei N, Rodd CJ, Kremer R, Khavandgar Z, Murshed M, Weiler HA. 2014. Dietary Vitamin D during pregnancy has dose-dependent effects on long bone density and architecture in guinea pig offspring but not the sows. Journal of Nutrition 25:1985–1994.
50. Tojo Y, Kurabayashi T, Honda A, Yamamoto Y, Yahata T, Takakuwa K, Tanaka K. 1998. Bone structural and metabolic changes at the end of pregnancy and lactation in rats. American Journal of Obstetrics and Gynecology 178:180–185.
51. Ulrich U, Miller PB, Eyre DR, Chesnut CH, Schlebusch H, Soules MR. 2003. Bone remodeling and bone mineral density during pregnancy. Archives of Gynecology and Obstetrics 268:309–316.
52. Vajda E G, Bowman BM, Miller SC. 2001. Cancellous and cortical bone mechanical properties and tissue dynamics during pregnancy, lactation, and postlactation in the rat. Biology of reproduction 65:689–95.
53. Vajda EG, Kneissel M, Muggenburg B, Miller SC. Increased intracortical bone remodeling during lactation in Beagle Dogs. 1999. Biology of Reproduction 61:1439–1444.
54. Verhaeghe J, Allewaert K, van Herck E, Van Bree R, Van Assche FA, Bouillon R. 1994. 1,25-Dihydroxyvitamin D3 and osteocalcin in maternal and fetal guinea pigs. Bone and Mineral 26:261–73.
55. Verhaeghe J, van Herck E, Van Bree R, Van Assche FA, Bouillon R. 1989. Osteocalcin during the reproductive cycle in normal and diabetic rats. Journal of Endocrinology 120:143–51.
56. Weber GM, Witschi AKM, Wenk C, Martens H. 2014. Effects of dietary 25-hydroxycholecalciferol and cholecalciferol on blood vitamin D and mineral status, bone turnover, milk composition, and reproductive performance of sows. Journal of Animal Science 92:899–909.
57. Woodrow JP, Sharpe CJ, Fudge NJ, Hoff AO, Gagel RF, Kovacs CS. 2006. Calcitonin plays a critical role in regulating skeletal mineral metabolism during lactation. Endocrinology 147:4010–4021.
58. Yildiz M, Oral B. 2006. The effects of pregnancy and lactation on bone mineral density in fluoride-exposes rats. Toxicology and Industrial Health 22:217–222.
59. Zeni SN, Di Gregorio S, Mautalen C. 1999. Bone mass changes during pregnancy and lactation in the rat. Bone 25:681–685.
60. Zeni S, Weisstaub A, Di Gregorio S, De Ferrer PR, De Portela ML. 2003. Bone mass changes in vivo during the entire reproductive cycle in rats feeding different dietary calcium and calcium/phosphorus ratio content. Calcified Tissue International 73:594–600.

Article 2: **Fruit-eating bats (*Artibeus lituratus*) lose bone tissue during reproduction but bone strength is not affected**

ABSTRACT

During the pregnancy and lactation of mammals, the growth and calcification of offspring increase the maternal demand of calcium. In addition to dietary calcium, females use bone calcium to supply this demand, leading to a decrease of bone mineral density and content. In reproductive insectivorous bats, the loss of bone has been hypothesized to reduce the fitness of females in terms of survival due to decreased bone mineral content, increasing the risk of bone fracture. However, the effect of pregnancy and lactation on the risk of bone fractures in bats or whether other non-insectivorous bats also lose bone tissue during reproduction remains unknown. In the present study, we examined the changes in bone turnover during pregnancy and lactation in the fruigivorous bat *Artibeus lituratus*. Additionally, we tested whether pregnancy and lactation reduce the strength of the humerus. Trabecular bone area females was decreased in lactating compared to pregnant and non-reproductive females. The porosity of diaphysis compact bone was not different between reproductive groups. The hypothesis that reproduction is a period of high risk of bone fracture was not supported due to the fact that bone mechanic parameters were not different between reproductive and non-reproductive females.

Keywords: Bone loss, Bone turnover, calcium, Chiroptera, lactation, Phyllostomidae, pregnancy.

INTRODUCTION

Litter size and the number of litters per year are life-history traits that vary greatly in mammals and other vertebrates. Body size is an important factor influencing the life-history variation in mammals (Sibly and Brown 2007). Small species tend to mature early, have short pregnancies, delivery larger litters and live short periods, whereas larger species mature slowly, have longer pregnancies, delivery smaller litters and live longer (Promislow and Harvey 1990; Bielby et al. 2007). Bats are the second largest group of mammals, and despite their physiological and ecological diversity, they exhibit little variation in litter size and number of litters per year (Barclay and Harder 2003). Most bats have one or two litters per year and give birth to one pup per litter, a remarkable pattern considering that bats have small body sizes. Other small mammals of similar size such as rodents or shrews have shorter pregnancy and lactation lengths, deliver larger litters and produce more pups per year than do bats (Barclay 1994; Barclay and Harder 2003).

The calcium constrain hypothesis as an explanation for the low reproductive rates of bats has recently received attention (Booher 2008; Booher and Hood 2010). Since calcium is the main compound of the skeleton and is essential for fetal and postnatal development, this hypothesis states that limitations of calcium due to diets with low calcium content and longer and stressful periods of lactation limit the number of offspring (Barclay 1994). Young bats are nutritionally dependent on their mother for long periods of time, due to their need to reach 70-80% of adult size to withstand the stress of flight (Hood et al. 2011), while other similar sized terrestrial mammals are nutritionally independent when they reach about 40% of their adult size (Barclay 1994). This implies that female bats invest more energy

and nutrients during lactation in each offspring than similar sized terrestrial mammals, which might limit large litter sizes.

In addition to dietary calcium, mammals also use the calcium stored in their bones to meet the demands of pregnancy and lactation (Bowman and Miller 2001, Kovacs 2016). If dietary calcium is low, the female's dependence on endogenous sources will be greater, increasing the loss of bone tissue (Schmidt and Hood 2014; Zeni et al. 2003). In bats, nutritional analysis of insects consumed by the insectivorous species Eptesicus fuscus revealed that level of calcium that a pregnant female consumes is 10 times below the estimated requirements (Keeler and Studier 1992). Considering this information, insect-eating female bats are likely to mobilize bone calcium reserves during lactation. Calcium mobilization from bone tissue seems to reduce the total body calcium concentration during lactation in this insectivorous bat (Booher and Hood 2010). The same pattern of bone mobilization phenomenon was observed in other insectivorous bats, such as *Myotis lucifugus* (Kwiecinski et al. 1987) and *Miniopterus schreibersii* (Bernard and Davison 1996), which also lose bone calcium during lactation.

Loss of calcium in the bone observed in insectivorous bats during pregnancy and lactation probably decrease their fitness (Kwiecinski et al. 1987; Barclay 1994) because the loss of minerals from bones decreases the bone strength and increases the risk of fracture (Turner 2006; Sanz-Salvador et al. 2014). A fractured bone might seriously affect the effectiveness of foraging and the ability to avoid predators, reducing the probabilities of survival and future reproduction (Speakman, 2008). According to this hypothesis, if pregnancy and lactation decrease the chances of survival, the bats litter size might be the maximum reproductive capacity that a female can attain without jeopardizing their survival

(i.e. trade-off reproduction/survival). Whether reproduction is a high risk period for fracture in bats, or whether other bats exhibit the pattern of bone loss shown by insectivorous bats remains unknown. In this study, we examine the changes in bone turnover during pregnancy and lactation in the fruigivorous bat *Artibeus lituratus*. We also test the hypothesis that reproduction increases the risk of bone fracture in female bats. For analysis, we selected the humerus, the most calcified bone of the bat wing (Papadimitriou and Kunz 1996).

MATERIALS AND METHODS

Bat sampling and tissues processing

Adult females (*A. lituratus*) were captured using mist-nets at night in two fragments of Atlantic forest (fragment 1: 20°45'22.89''S 42°51'48.1''W; fragment 2: 20°48'09.5''S 42°51'32.45''W) from October 2015 to September 2016, in the municipality of Viçosa, Minas Gerais State, Brazil. Captured females were taken to the Laboratory of Experimental Pathology of the Federal University of Viçosa for tissue collection. The euthanasia was performed in the following morning by decapitation (Reeder and Widmaier, 2009). National environmental authorities approved animal's capture and transportation (license number 50517-1 SISBIO) and the Animal Use and Care Committee of the Federal University of Viçosa approved all the procedures (CEUA/UFV – number 89/2015).

To ensure adult status, only animals with complete ossification of the cartilaginous epiphyseal growth plates of metacarpal phalangeal joints were selected (Brunet-Rossinni and Wilkinson, 2009). Females were allocated into four groups: (1) pregnancy, when a fetus was identified by abdominal palpation; (2) lactation, when the nipples were producing milk

and the surrounded area was lacking fur; (3) post-lactating, when nipples were similar to those of lactation but were not producing milk and the area around the nipple was recovering its fur; and (4) inactive, when the adult female was not assigned to the first three categories.

Blood was collected from the trunk into heparinized tubes, centrifuged and stored at -80 °C for minerals analyses. Both humeri were extracted, cleaned and weighted. The left humerus was wrapped in gauze, soaked in saline solution and stored at -20 °C for bone strength analyses. The right humerus was cut into two equal portions. The proximal portion was fixed in neutral buffered formalin for 48 hours and then transferred to 70% ethanol for morphometric analyses. Distal portion was stored in 70% ethanol for scanning electron microscopy (Boyde, 2012).

Bone histomorphometric analyses

The proximal portion of the humeri were immersed in a decalcifying solution of formic acid and sodium citrate for 30 days (Morse 1945). Decalcified bones were cut longitudinally to expose the trabecular bone of the epiphysis. Bone fragments were then dehydrated in ethanol, cleared with xylene, embedded in paraffin and cut in sections of five µm of thickness. These sections were prepared as histological slides and stained with hematoxylin and eosin. We randomly selected and digitalized 10 regions of interest (ROI) by each female using the 10x objective lens in light microscope (Olympus BX-60 ®, Tokyo, Japan) integrated with a digital camera (Olympus QColor-3 ®, Tokyo, Japan). Each ROI represented one area of the 36x104 µm2 of trabecular bone. In order to estimate bone area (B.Ar) in each ROI, we deleted the marrow, considering only the trabeculae, and

transformed the ROI in a black and white image (trabeculae was black and the background was white). Then, we calculated how much of the ROI were covered by trabeculae (Egan et al. 2012). Black and white images were also used to estimate trabecular width (Tb.Wi) and trabecular separation (Tb.Sp) using the spheres fitting method. This method uses the average of the diameters of all spheres that can be fit in the trabeculae (Tb.Wi) or between the trabeculae (Tb.Sp) (Hildebrand and Rüegsegger, 1997). Analyses were run using the software ImageJ (Schneider et al. 2012) with the plugin BoneJ (Doube et al. 2010).

Bone strength test

Left humeri were removed from storage at -20 °C and left at room temperature three hours prior to test. A three point bending test using a universal testing machine (Instron® 3367) was performed to test the bone strength. We applied a force on the middle third (diaphysis) of the humeri using a load cell of 250 Newton at a speed of 10 mm/s until bone fracture. We record the maximum force and displacement at fracture. We also calculated the bone stiffness as the slope in the linear region of the load-displacement curve.

Blood and bone minerals analysis

We analyzed serum calcium and phosphorus concentrations using colorimetric tests (Bioclin, Brazil). After bone strength test, left humeri were dried at 70 ºC until they reached constant weight. Bones were homogeneously macerated and two grams of each bone were digested in a 5 mL solution of nitric acid and perchloric acid at 200 °C. Then, we completed to a volume of 10 mL with distilled water and concentrations of calcium and phosphorus were determined by atomic absorption spectrometry.

Scanning electron microscopy of diaphysis

Diaphysis fragments were immersed in Tergazyme (Alconox Inc., White Plains, NY, USA) for 30 days at 40 °C to remove organic tissue (Boyde 2012). Fragments were dehydrated in absolute alcohol and allowed to dry at room temperature. Fragments were coated with gold and analyzed using scanning electron microscopy (LEO 1430VP, Zeiss, Oberkochen, Germany). We captured two ROI by female at 2000x of magnification. Each ROI represented 15x103 μm2 of compact bone surface. In each ROI, we established a grid of 368 points and calculated the proportion of points that overlay bone pores.

Statistical analysis

In order to test data normal distribution, we used Shapiro-Wilk test. Since all data sets were normally distributed, we used one-way ANOVA to test differences between reproductive groups. When differences existed, we used t-student test to compare all pairs. Simple linear regression was used to test the relation between the percentage of pores on diaphysis and bone strength parameters. Analyses were run in the statistical software R. Results were expressed as mean ± SD, with a significance level of 5% ($P<0.05$).

RESULTS

We capture in total thirty-two females. Nine females were inactive; eight were pregnant; nine were lactating and six were post-lactating. Body weight, excluding the fetus weight in pregnant females, were not different among groups (69.2 ± 6.1 g; $F = 1.10$, $P = 0.377$). Both right (0.56 ± 0.06 g; $F = 1.27$, $P = 0.30$) and left humeri (0.55 ± 0.05 g; $F = 0.91$, $P =$

0.45) weight were also not different among groups.

Serum calcium (9.64 ± 1.06 mg/dL; F = 3.69, P = 0.08) and phosphorus (3.79 ± 1.94 mg/dL; F = 1.10, P = 0.377) were not different among groups (Fig. 1A-B). Calcium bone content were also not different among groups (calcium: 9.17 ± 1.25 mg/g; F = 2.58, P = 0.078; Fig. 1C). Phosphorus bone content (Fig. 1D) was higher in postlactating females (0.36 ± 0.03 mg/g) compared to pregnant (0.30 ± 0.03 mg/g; T = 2.73, P = 0.023) and lactating females (0.31 ± 0.03 mg/g; T = -2.62, P = 0.023).

Trabecular bone area (Fig. 2) was decreased in lactating (23.3 ± 4.2 %) females compared to non-reproductive (29.5 ± 5.3 %; T = 2.56, P = 0.023), pregnant (30 ± 7 %; T = -2.29, P = 0.039) and postlactating females (33.9 ± 3.2 %; T = -5.16, P < 0.01) (Fig. 3A). Trabecular width and trabecular separation (Fig. 2) were not different among groups (thickness: 71.7 ± 17 µm, F = 1.88, P = 0.16; separation: 205.8 ± 43.8 µm, F = 1.09, P = 0.372) (Fig. 3B-C). Percentage of pores on diaphysis surface (Fig. 4) was similar among groups (23.5 ± 8.8 %, F = 3.42, P = 0.064) (Fig. 3D).

Maximum load at fracture was not different among groups (F = 1.31, P = 0.289) (Fig. 5A). Maximum displacement at fracture (F = 2.31, P = 0.098) and bone stiffness (F = 0.73, P = 0.542) also did not differ among groups (Fig. 5B-C). The percentage of pores did not explain significantly the variation of maximum load at fracture (R2 = 0.008, T = 0.32, P = 0.748), maximum displacement at fracture (R2 = 0.082, T = 1.08, P = 0.298) and bone stiffness (R2 = 0.001, T = 0.13, P = 0.896).

DISCUSSION

In this study, we report for the first time the loss of bone during lactation in a fruigivorous

bat. Bone loss was confirmed by the decrease of trabecular bone area in the epiphysis of the humerus during lactation. This tissue loss, however, seems to be minimal and not affect bone structural parameters such as trabecular thickness and separation. The hypothesis that reproduction is a period of high risk of bone fracture in female bats was not supported since bone strength parameters were not decreased in pregnant and lactating groups. The surface of compact bone at diaphysis also seems unaffected, as porosity did not vary among groups.

Calcium is the main mineral in mammals' bone tissues (Doherty et al. 2015). Its constant release into the blood through osteoclastic bone resorption ensure the increase of calcium concentration when it drops below the required physiological levels (Gonciulea and Jan de Beur 2015). Many species of mammals resorb bone during pregnancy and lactation to compensate for the continuous loss of calcium through the placenta and mammary glands for offspring supply (Ulrich et al 2003, Liu et al. 2012). In our study, we observed that *A. lituratus* also reabsorb bone tissue during lactation, suggesting that females probably use bone calcium to meet the demands of reproduction.

Total serum calcium concentration found in *A. lituratus* was found to be similar to values from other species of mammals, including frugivorous bats species (Korine et al. 1999; Heard et al. 2006). This concentration in pregnant and lactating females is usually lower than in non-reproductive females (Weber et al. 2014, Kovacs 2016). As we did not find lower blood calcium concentration in reproductive females, we suggest that bone calcium mobilization in *A. lituratus* is sufficient to maintain calcium homeostasis during reproduction.

Calcium demand during lactation is especially high, since the newborn acquires calcium at higher rates during this period when compared to pregnancy (Kovac 2011). In

addition, higher prolactin and parathyroid hormone-related protein concentrations during lactation induce bone resorption by stimulating osteoclasts, releasing more calcium into the bloodstream (Charoenphandhu et al. 2010; Wysolmerski 2012). The pattern of bone loss found in *A. lituratus* corroborates this view, since lactating females lost more bone tissue than pregnant females, similarly to that found in insectivorous bats (Kwiecinski et al. 1987; Bernard and Davison 1996).

The bone tissue is formed by an inorganic part, minerals, and an organic part, formed mainly by type I collagen. The mineral component gives the bone strength and stiffness (Augat and Schorlemmer 2006; Turner 2006). A decrease in bone mineral components is usually associated with a decrease in bone tissue resistance to fractures (Wachter 2002). Our study demonstrated a decrease in bone tissue of *A. lituratus* females, but there was no decrease in bone resistance, suggesting that the amount of calcium lost during reproduction is not sufficient to cause tissue strength to decrease. The bone strength may also not be affected since the calcium loss was concentrated mainly at epiphysis and probably less at diaphysis, place where we quantified bone strength. The loss of trabecular bone but not the increase of cortical porosity supports this view.

Studies demonstrating calcium supplementation in mammals during lactation have shown that supplementation with this mineral is in general not sufficient to reduce bone loss, demonstrating that this may be an inherent physiological cost of lactation (Zeni et al. 2003; Taylor et al. 2009). The possible explanation for this cost would be that the physiological environment of lactation is incompatible with the retention of calcium in bone (Speakman 2008), since hormone-driven processes would take place i.e. parathyroid hormone-related protein stimulate bone resorption by osteoclasts. This cost is more severe

when dietary calcium is low (Glade 1993; Gruber and Stover 1994; Zeni et al. 2003). *A. lituratus* feed preferentially on fig fruits (Andrade et al. 2013), which have high calcium content (Wendeln et al. 2000), and occasionally consume leaves rich in calcium (Zortéa and Mendes 1993; Nelson et al. 2005), suggesting that *A. lituratus* reproductive females are probably not highly limited by dietary calcium and the magnitude of bone loss is minimum, only reflected in decreased bone area but not sufficient to affect the structural bone parameters such as trabecular thickness and trabecular separation.

In mammals, the bone tissue lost during lactation is rapidly recovered after weaning, due to an increase of bone formation and a decrease of bone resorption (Ardeshirpour 2007; Liu et al. 2012). This post-weaning is characterized by very high rates of bone formation, and sometimes the females reach a higher bone mineral density than they had before reproduction (Miller et al. 2005). We found that *A. lituratus* also recovered from lactation and post-lactating females showed a bone area slightly higher (34%) than non-reproductive females (29%). We also found that post-lactating females showed higher concentrations of phosphorous in bone than pregnant and lactating females, suggesting a possibly accretion of bone hydroxyapatite crystals, which are formed largely by phosphorus.

We conclude that *A. lituratus* showed a pattern of bone tissue mobilization during reproduction similar to insectivorous bats and other mammals. However, this loss seems to not affect bone strength during this period. Our results did not support the hypothesis of reproduction as a time of high bone fracture risk, which challenge the idea that smaller litter size in bats is probably influenced by a trade-off between reproduction and survival. In future studies, it will be interesting to investigate whether species that raise more than one pup at one time (e.g. Lasiurus spp.) or whether species with low dietary calcium contents

(e.g. nectarivorous bats) showed similar patterns of bone change and strength.

ACKNOWLEDGMENTS

We thank to Esteban Giraldo, David Justinico, Evaristo Villalba and Nicole Fontes, who helped us during field and laboratory work. We are also grateful to Sergio da Matta and Gisele Lessa for allowing us access to their laboratories and equipment. This work is part of the postgraduate dissertation of D. A. Torres, who was funded by the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) of Brazil.

REFERENCES

Andrade TY, Thies W, Rogeri PK, Kalko EKV, Mello MAR. 2013. Hierarchical fruit selection by Neotropical leaf-nosed bats (Chiroptera: Phyllostomidae). Journal of Mammalogy 94:1094–1101.

Ardeshirpour L, Dann P, Adams DJ, Nelson T, VanHouten J, Horowitz MC, Wysolmerski JJ. 2007. Weaning Triggers a Decrease in Receptor Activator of Nuclear Factor-κB Ligand Expression, Widespread Osteoclast Apoptosis, and Rapid Recovery of Bone Mass after Lactation in Mice. Endocrinology 148:3875–3886.

Barclay R, Harder ML. 2003. Life histories of bats: life in the slow lane. Pp. 209–253 in Bat ecology (Kunz TH, Fenton MB, eds.). University of Chicago Press, Chicago.

Barclay R. 1994. Constraints on reproduction by flying vertebrates: Energy and Calcium. The American Naturalist 144:1021.

Bernard RTF, Davison A. 1996. Does calcium constrain reproductive activity in insectivorous bats? Some empirical evidence for Schreibers' long-fingered bat (Miniopterus

schreibersii). South African Journal of Zoology 31:218–220.

Bielby J, Mace GM, Bininda-Emonds ORP, Cardillo M, Gittleman JL, Jones KE, Orme CDL, Purvis A. 2007. The fast-slow continuum in mammalian life history: an empirical reevaluation. American Naturalist 169:748–757.

Booher C, Hood W. 2010. Calcium utilization during reproduction in big brown bats (Eptesicus fuscus). Journal of Mammalogy 91:952–959.

Booher C. 2008. Effects of calcium availability on reproductive output of big brown bats. Journal of Zoology 274:38–43.

Bowman BM, Miller SC. 2001. Skeletal adaptations during mammalian reproduction. Journal of musculoskeletal and neuronal interactions 1:347–355.

Boyde A. 2012. Scanning electron microscopy of bone. Pp. 365–400 in Bone research protocols (Helfrich M, Ralston H, eds.). Humana Press, Totowa.

Brunet-Rossinni AK, Wilkinson GS. 2009. Methods for age estimation and the study of senescence in bats. Pp. 315–328 in Ecological and behavioral methods for the study of bats (Kunz TH, Parsons S, eds.). The Johns Hopkins University Press, Baltimore.

Charoenphandhu N, Wongdee K, Krishnamra N. 2010. Is prolactin the cardinal calciotropic maternal hormone? Trends in endocrinology and metabolism 21:395–401.

Doherty A, Ghalambor CG, Donahue SW. 2015. Evolutionary physiology of bone: bone metabolism in changing environments 30:17–29.

Doube M, Klosowskia MM, Arganda-Carreras I, Cordelières FP, Dougherty RP, Jackson JS, Schmidf B, Hutchinsong JR, Shefelbine SJ. 2010. BoneJ: free and extensible bone image analysis in ImageJ Michael. Bone 47:1076–1079.

Egan KP , Brennan TA, Pignolo RJ. 2012. Bone histomorphometry using free and

commonly available software. Histopathology 61:1168–1173.

Glade MJ. 1993. Effects of Gestation, Lactation, and maternal calcium intake on mechanical strength of equine bone. Journal of the American College of Nutrition 12:372–377.

Gonciulea A, Jan de Beur S. 2015. The dynamic skeleton. Reviews in Endocrine and Metabolic Disorders 16:79–91.

Gruber HE, Stover SJ. 1994. Maternal and weanling bone: The influence of lowered calcium intake and maternal dietary history. Bone 15:167–176.

Heard DJ, Ruiz M, Harr KE. 2006. Comparison of serum and plasma for determination of blood biochemical values in Malaysian Flying Foxes (*Pteropus Vampyrus*). Journal of Zoo and Wildlife Medicine 37:245–248.

Hildebrand T, Rüegsegger P. 1997. A new method for the model-independent assessment of thickness in three-dimensional images. Journal of Microscopy 185:67–75.

Hood W, Oftedal OT, Kunz TH. 2011. Is tissue maturation necessary for flight? Changes in body composition during postnatal development in the big brown bat. Journal of Comparative Physiology B 181:423–435.

Keeler JO, Studier EH. 1992. Nutrition in pregnant big brown bats (Eptesicus fuscus) feeding on june beetles. Journal of Mammalogy 73:426–430.

Korine C, Zinder O, Arad Z. 1999. Diurnal and seasonal changes in blood composition of the free-living Egyptian fruit bat (*Rousettus aegyptiacus*). Journal of Comparative Physiology - B Biochemical, Systemic, and Environmental Physiology 169:280–286.

Kovacs CS. 2011. Calcium and bone metabolism disorders during pregnancy and lactation. Endocrinology and Metabolism Clinics of North America 40:795–826.

Kovacs CS. 2016. Maternal mineral and bone metabolism during pregnancy, lactation and post-weaning recovery. Physiological Reviews 96:449-547.

Kwiecinski GG, Krook L, Wimsatt WA. 1987. Annual skeletal changes in the little brown bat, *Myotis lucifugus lucifugus*, with particular reference to pregnancy and lactation. The American journal of anatomy 178:410–420.

Liu XS, Ardeshirpour L, Vanhouten JN, Shane E, Wysolmerski JJ. 2012. Site-specific changes in bone microarchitecture, mineralization, and stiffness during lactation and after weaning in mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 27:865–875.

Miller SC, Anderson BL, Bowman BM. 2005. Weaning initiates a rapid and powerful anabolic phase in the rat maternal skeleton. Biology of Reproduction 73:156–162.

Morse, A. 1945. Formic acid-sodium citrate decalcification and butyl alcohol dehydration of teeth and bones for sectioning in paraffin. Journal of Dental Research 24:143–153.

Nelson SL, Kunz TH, Humphrey SR. 2005. Folivory in fruit bats: leaves provide a natural source of calcium. Journal of Chemical Ecology 31:1683-1691.

Papadimitriou HM, Swartz SM, Kunz TH. 1996. Ontogenetic and anatomic variation in mineralization of the wing skeleton of the Mexican free-tailed bat, *Tadarida brasiliensis*. Journal of Zoology 240: 411-426.

Peacock, M. 2010. Calcium metabolism in health and disease. Clinical Journal of the American Society of Nephrology 5:23–30.

Promislow DEL, Harvey PH. 1990. Living fast and dying young: A comparative analysis of life-history variation among mammals. Journal of Zoology 220:417–437.

Reeder D, Widmaier E. 2009. Hormone Analysis in Bats. Pp. 554–563 in Ecological and

Behavioral Methods for the Study of Bats (Kunz TH, Parsons S, eds.). The Johns Hopkins University Press, Baltimore.

Sanz-Salvador L, Garcia-Perez MA, Tarin JJ, Cano A. 2014. Endocrinology in pregnancy: bone metabolic changes during pregnancy: a period of vulnerability to osteoporosis and fracture. European Journal of Endocrinology 172:53–65.

Schmidt CM, Hood W. 2014. Bone loss is a physiological cost of reproduction in white-footed mice (*Peromyscus leucopus*). Mammalian Biology 79:96–100.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9:671–675.

Sibly RM, Brown JH. 2007. Effects of body size and lifestyle on evolution of mammal life histories. Proceedings of the National Academy of Sciences 104:17707–17712.

Speakman JR. 2008. The physiological costs of reproduction in small mammals. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 363:375–398.

Taylor MS, Knowlton KF, McGilliard ML, Swecker WS, Ferguson JD, Wu Z, Hanigan MD. 2009. Dietary calcium has little effect on mineral balance and bone mineral metabolism through twenty weeks of lactation in Holstein cows. Journal of dairy science 92:223–37.

Turner CH. 2006. Bone strength: Current concepts. Annals of the New York Academy of Sciences 1068:429–446.

Ulrich U, Miller PB, Eyre DR, Chesnut CH, Schlebusch H, Soules MR. 2003. Bone remodeling and bone mineral density during pregnancy. Archives of Gynecology and Obstetrics 268:309–316.

Wachter NJ, Krischak GD, Mentzel M, Sarkar MR, Ebinger T, Kinzl L, Claes L, Augat P. 2002. Correlation of bone mineral density with strength and microstructural parameters of cortical bone in vitro. Bone 31:90–95.

Weber GM, Witschi KM, Wenk C, Martens, H. 2014. Effects of dietary 25-hydroxycholecalciferol and cholecalciferol on blood vitamin D and mineral status, bone turnover, milk composition, and reproductive performance of sows. Journal of Animal Science 92:899–909.

Wendeln MC, Runkle JR, Kalko EKV. 2000. Nutritional values of 14 fig species and bat feeding preferences in Panama. Biotropica 32:489–501.

Wysolmerski, JJ. 2012. Parathyroid Hormone-Related Protein: An Update. The Journal of Clinical Endocrinology & Metabolism 97:2947–2956.

Zeni S, Weisstaub A, Di Gregorio S, De Ferrer PR, De Portela ML. 2003. Bone mass changes in vivo during the entire reproductive cycle in rats feeding different dietary calcium and calcium/phosphorus ratio content. Calcified Tissue International 73:594–600.

Zortea M, Mendes SL. 1993. Folivory in the Big Fruit-Eating Bat, *Artibeus lituratus* (Chiroptera: Phyllostomidae) in Eastern Brazil. Journal of Tropical Ecology 9:117-120.

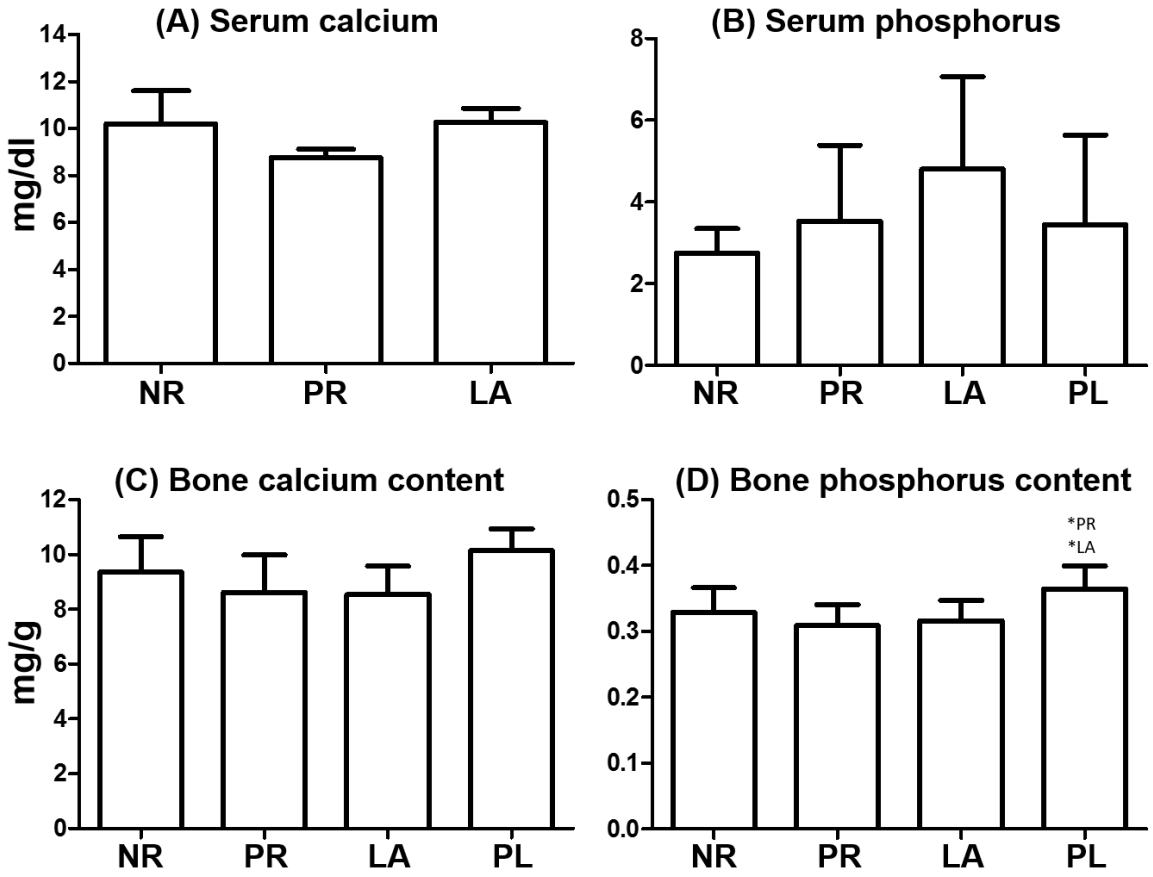


Figure 1. Calcium (A) and phosphorus (B) content in the serum. Calcium (C) and phosphorus (D) content in the bone as mg/g of dry bone. * $p<0.05$. NR = non-reproduction, PR = pregnancy, LA = lactation, PL = post-lactation.

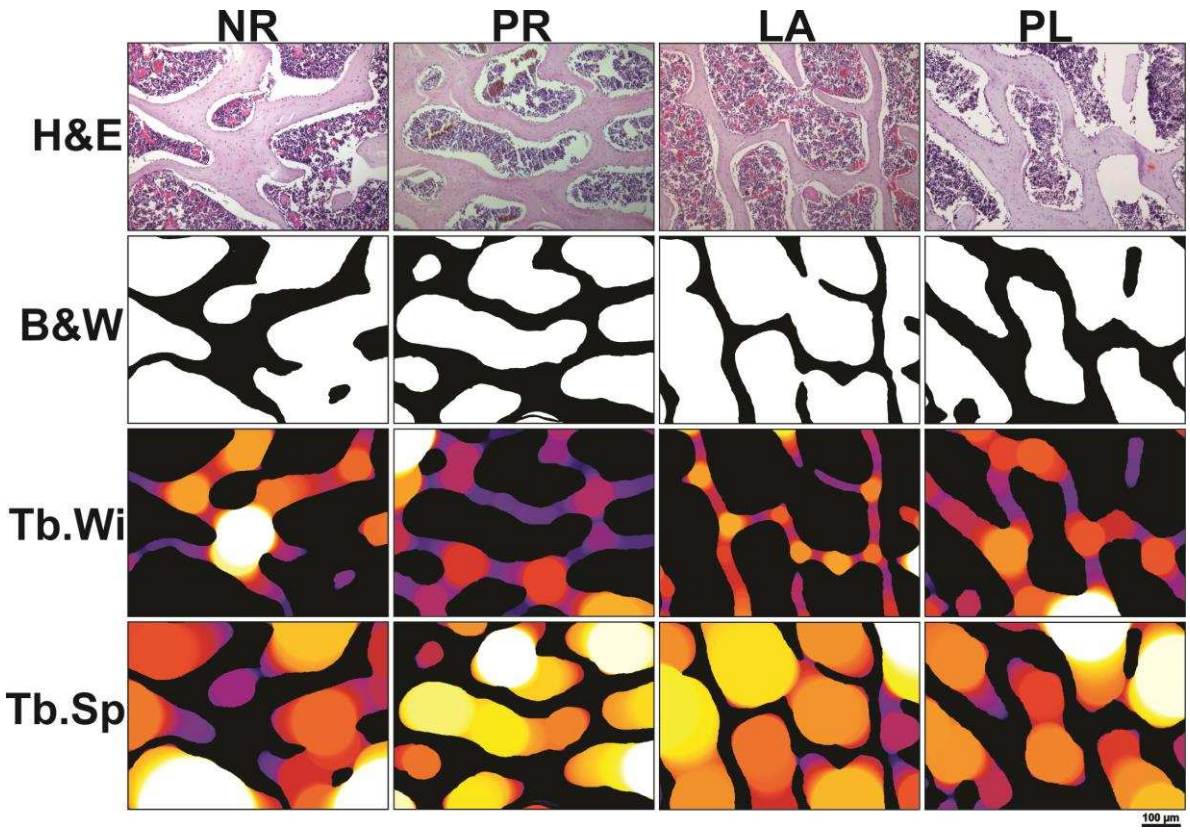


Figure 2. (H&E): histological section of trabecular bone stained with Hematoxylin and eosin. (B&W): black (trabeculae) and white (background) images of trabecular bone. (Tb.Wi): quantification of trabecular width by the sphere fitting method in the white and black images of a non-reproductive female. (Tb.Sp): quantification of trabecular separation by the sphere fitting method in the white and black images. NR = non-reproduction, PR = pregnancy, LA = lactation, PL = post-lactation.

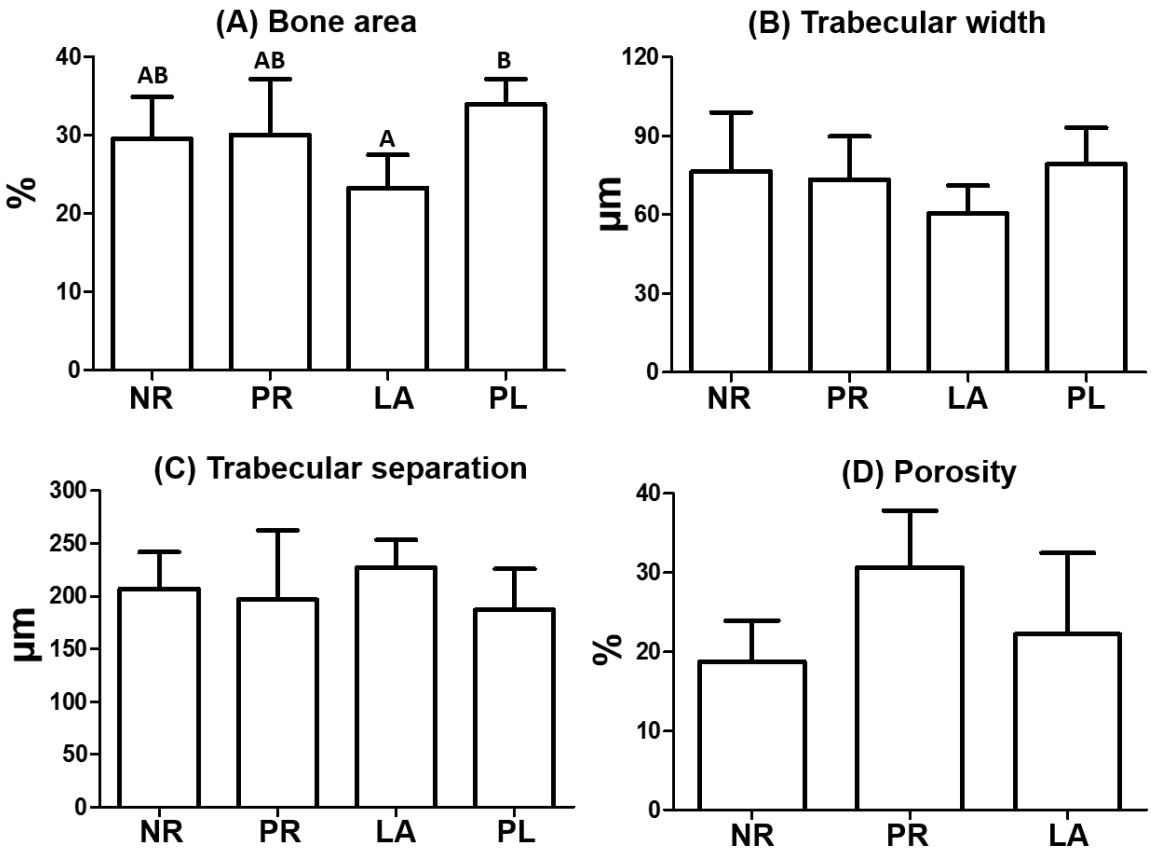


Figure 3. Bone area (A), trabecular width (B) and trabecular separation (C) in the epiphysis of the humeri. Porosity (D) in the surface of the diaphysis. * $p<0.05$, ** $p<0.01$. NR = non-reproduction, PR = pregnancy, LA = lactation, PL = post-lactation.

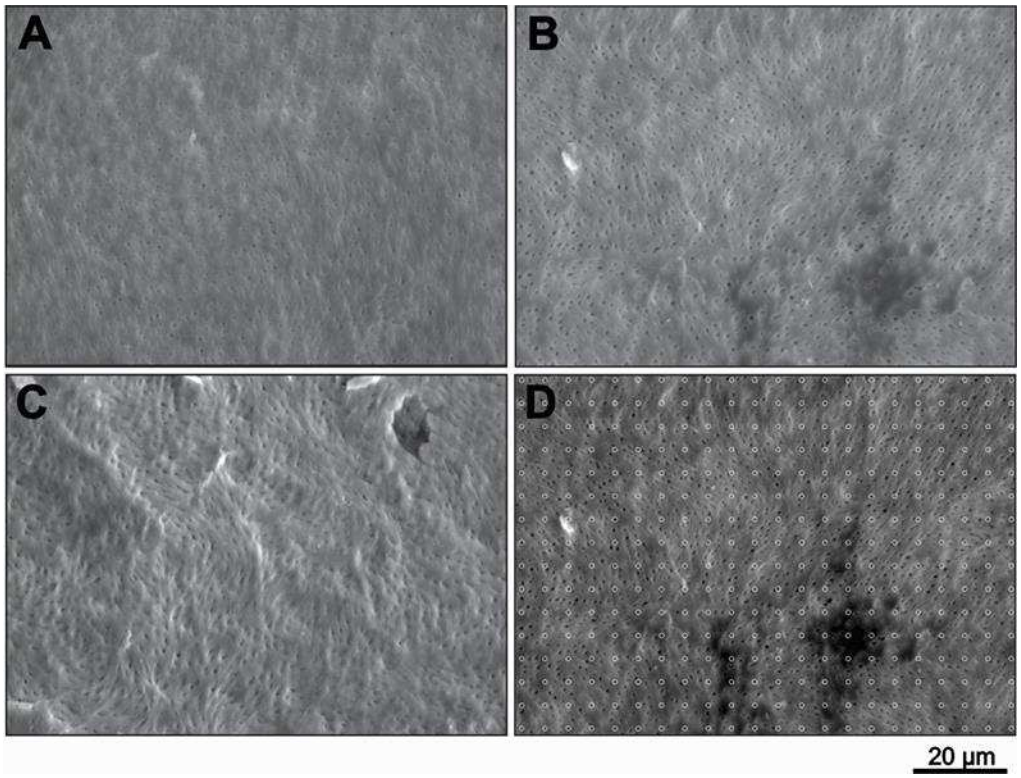


Figure 4. Scanning electron microscopy images of diaphysis surface at 2000x of magnification of a non-reproductive female (A), pregnant female (B) and lactating female (C). Image D illustrate the grid of 368 points used to quantify the number of pores.

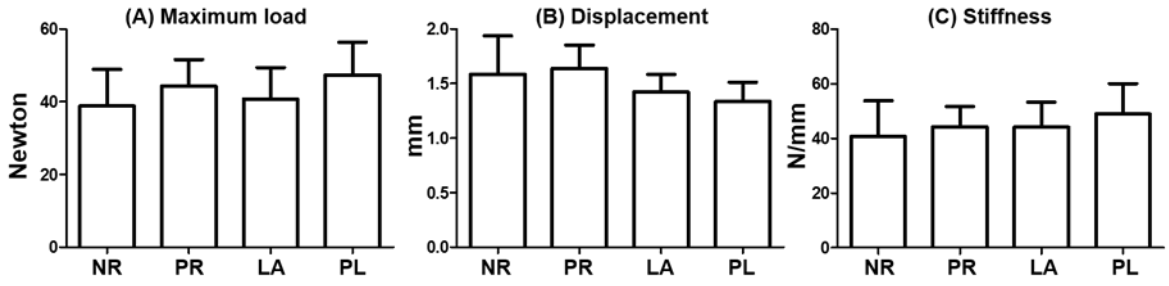


Figure 5. Maximum load (A) and displacement (B) at humeri fracture. Stiffness (C) as the slope of the linear region of the load/displacement curve. NR = non-reproduction, PR = pregnancy, LA = lactation, PL = post-lactation.