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The role of female reproductive history in determining variation in cortical bone remodeling and trabecular architecture in a nonhuman animal model (*Papio hamadryas*)

By

Ashley Nicole Lipps

A dissertation submitted in partial satisfaction of the

requirements for the degree of

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in

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in the

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University of California, Berkeley

Committee in charge:

Professor Sabrina Agarwal, Chair
Professor Terrence Deacon
Professor Marvalee Wake

Fall 2014

The role of female reproductive history in determining variation in cortical bone remodeling and trabecular architecture in a nonhuman animal model (*Papio hamadryas*)

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Abstract

The role of female reproductive history in determining variation in cortical bone remodeling and trabecular architecture in a nonhuman animal model (*Papio hamadryas*)

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This study investigates variance in bone quality and quantity with respect to site, sex, and female reproductive history in a model species, baboons. Three sites are examined: 1) the midshaft humerus, 2) the midshaft femur, and 3) the L1 vertebra. A total of 109 individuals are included in the humerus analyses, 76 in the femur analyses, and 24 in the lumbar analyses. Intracortical remodeling at the midshaft humerus and femur is assessed through static histomorphometry and trabecular architecture of the lumbar vertebra is assessed through micro-CT. Analyses of bone micro and macrostructural variance reveal significant trends.

Site-specific patterns are observed between the humerus and femur in baboons. Most notably: high porosity, percent osteonal bone, and low adjusted cortical area characterize the midshaft humerus as significantly more remodeled and porous than the midshaft femur. Significant sex differences are also observed. At the midshaft humerus, males grow larger osteons and Haversian canals but have lower overall activation of remodeling whereas females have increased secondary osteon activity (e.g. higher osteon population density, and percent osteonal bone) but exhibit smaller osteons and Haversian areas.

Some of the variance observed in females is related to reproductive history. At both the midshaft humerus and femur, osteon sizes are increased in high parity females, and this may be related the cumulative exposure to elevated estrogen levels during multiple pregnancies, as estrogen has been observed experimentally to increase osteoblast activity and decrease osteoclast functioning. Percent porosity at the midshaft humerus is also observed to be significantly higher in females with high lactation histories. Short-term cortical porosity during lactation has been observed in several animal model species (e.g. rat, dog), typically with only partial recovery. Here, the high porosity in females with high lactation histories suggests that full recovery from short-term lactation-related resorption is incomplete, resulting in increased porosity in the long-term. However, despite the increased intracortical porosity in high-lactation females, significant increases in total and cortical cross-sectional areas are seen at the humerus and femur. Increased periosteal apposition during pregnancy (or retrospectively with parity) at appendicular sites has also been observed in macaques, rats, and humans and here, the behavioral aspects of infant-rearing associated with long periods of juvenile care and nursing also appear to increase the outer

cortex (periosteum) of the humerus and femur. The inverse reproductive-specific relationship of periosteal deposition with increased endosteal resorption may have an adaptive mechanical significance. Endocortical resorption results in a decrease in mechanical strength of cortical bone and the increases in periosteal apposition and overall cross-sectional area may help protect the maternal skeleton from fracture during this catabolic period. As long as periosteal apposition continues, the effects of increased intracortical porosity should not have detrimental effects on overall cortical bone health and fracture. Therefore, the overall effects of high parity and lactation on appendicular cortical bone health seen here in the humerus and femur are theoretically neutral.

Analyses here also revealed a potentially interesting relationship between lactational history and lumbar trabecular architecture. The female with the highest lactation history (34% of her life was spent nursing) exhibits abnormally high trabecular spacing, and abnormally low trabecular number and connectivity. Although short-term pregnancy-related changes observed here as well as in rats and humans show increased trabecular number and connectivity, the large resorption of cancellous bone that occurs during multiple cycles of long lactation appear to deteriorate these "new" struts, leaving behind large spaces, a reduced number of trabeculae, and overall decreased connectivity. Although this particular female's trabecular spacing is high, and her connectivity is low, she exhibits a bone volume measurement in the normal range. Because bone volume has the most significant effects on bone strength and stiffness it is unclear if this outlier female's abnormal trabecular architecture puts her at a mechanical disadvantage. Therefore, the long-term effects of reproductive differences at the lumbar site studied here also appear to be neutral. However, both the short and long-term alterations observed in this study are based on a small number (n=3) of outliers and need to be more rigorously tested in a larger sample group.

According to evolutionary theory, females in an iteroparous species that produce multiple offspring over time (e.g. monkeys and humans) would have selectively evolved mechanisms to maintain the skeleton over an entire lifetime of reproduction to increase reproductive success and optimal offspring survival. If evolution favored mechanisms that conserve and repair skeletal tissues during these reproductive periods, there should be no or little detrimental effect to the skeleton in the long term. In this study, even though aspects of bone quality and quantity did show variance with respect to reproductive history, these changes likely do not have strong effects on bone fragility and fracture risk. Therefore, the overall effects of high parity and lactation on appendicular cortical bone health and trabecular quality and quantity seen here are theoretically neutral. However, mechanical testing was not directly carried out in this study. Future work investigating both cortical and trabecular micro-architectural changes with reproductive history should include mechanical testing of specimens to clarify how these specific combinations of microstructural and macrostructural alterations affect bone fragility and fracture risk.

The approaches used in this dissertation advance biological anthropology and bone science by implementing thorough examinations of age, sex, and female reproductive history with respect to micro and macrostructural difference in cortical and trabecular bone. This study also contributes methodologically by demonstrating the importance of using multiple lines of evidence when exploring variance in bone quality and quantity.

For my loving husband and our sweet "old pup,"

for making every day a happy one

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right-hand woman during the grueling and frustrating work associated with prepping and coring all of the lumbar vertebrae. The two of them (Andy and Neha) went well above and beyond the requirements of a typical lab volunteer and I am forever grateful for their dedication and commitment to the work. I also owe gratitude to several other students for their time and energy spent in the Skeletal Biology Laboratory: Nicole Pay, Wyatt Lienhard, Layne Bernstein, Corey Wood, and Brandt Champion. On the UC Davis campus, Tanya Garcia-Nolen generously assisted me with my micro-CT scanning and data transformation.

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List of Abbreviations

Midshaft Cortical Histomorphometry
H.Ar — Haversian Area

On.Ar — Osteon Area

Dh.Ar — Drifter Haversian Area

DOn.Ar — Drifter Osteon Area

%On.B — Percent Osteonal Bone

%Po.Ar — Percent Porosity

OPD — Osteon Population Density

%C/T — Percent Cortical to Total Area

%%C_A/T — Percent Cortical Area (Corrected for Porosity)

Midshaft Cross-Sectional Area
Tt.Ar- Total Area

Ct.Ar — Cortical Area

Es.Ar — Endosteal Area

Lumbar Trabecular Architecture
BV/TV — Bone Volume

Tb.N — Trabecular Number

Tb.Sp — Trabecular Spacing

Tb.Th — Trabecular Thickness

ConnD — Connective Density

SMI- Structural Model Index

Reproductive History Variables
Parity — Total Number of Live Offspring

IBIavg — Average Interbirth Interval

%LifeLact — Percentage of Lifetime Spent Lactating

Chapter 1

Introduction

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Human and non-human skeletal tissues are uniquely plastic, dynamic, and reflect intricate relationships between phylogeny, ontogeny, mechanics, and individual factors (genotype-by-environment, diet, health). At the microstructural level, cortical bone remodeling varies by vertebrate taxa, and within individuals by age and skeletal element examined (Enlow and Brown, 1958; Enlow, 1962a; Kerley, 1966; Shaffler and Burr, 1985, Paine, 1994; Currey, 2002; Lieberman et al., 2003; Warshaw, 2007; McFarlin et al., 2008). Recent work has primarily focused on the biomechanical significance of remodeling patterns (Enlow, 1962b; Martin et al., 1998; Currey, 2002) as well as genetic (Hansen et al., 2009; Havill et al. 2010, 2013) and phylogenetic and ontogenetic effects (Warshaw, 2007; McFarlin et al., 2008). However, the inherent complexity of the factors relating to overall bone tissue organization and structural integrity makes studying and interpreting population and species-specific skeletal variation particularly challenging.

One essential function of bone remodeling is assisting in calcium homeostasis. In reproducing women, pregnancy and lactation are periods of significant changes to calcium needs, and the dramatic physiological fluctuations during these periods are associated with significant alterations to osteoblastic and osteoclastic activity in skeletal tissues (Zeni et al., 2003, Naylor et al., 2003). Pregnancy and lactation are also periods of increased maternal-fetal calcium transfer, and although changes to maternal calcitropic hormone activity levels aid in the absorption and retention of calcium from dietary sources, the maternal skeleton contributes a significant portion of calcium from its stores during these periods (Kovacs, 2001; 2005). At birth, a human neonate has accumulated approximately 30g of calcium, and at weaning, an addition 75g as the skeleton continues to mineralize during breastfeeding (Kovacs 2001; 2005). This places a significant demand on maternal mineral stores and temporary bone mass losses can average -4.5% during pregnancy and -7.5% during lactation, depending on the individual and skeletal site studied (Olausson et al., 2012). These bone mass losses are typically only temporary and are reversed some time after weaning and resumption of menses (Kalkwarf and Specker, 1995, Kolthoff et al., 1998, Lopez et al., 1996, Sowers et al., 1993). However, the lasting re-organizing effects of these active periods of bone resorption and subsequent rebuilding are not well understood. A recently growing body of literature in both human and non-human female primates finds significant changes to bone mass density (BMD) and risk of bone fracture in peri and post-menopausal age cohorts in relation to aspects of reproductive history, such as parity (number of offspring), average duration of lactation, and inter-birth interval (time between birthing successive offspring). In females, reproductive history may play a significant role in determining variation in bone remodeling and microstructure.

Women from both contemporary and past populations exhibit a large range of breastfeeding durations, ending after only a few hours to more than five years, spanning the range observed for all mammals (Hartmann and Arthur, 1986). Flexible lactation patterns allow mammalian mothers to respond to environmental and social factors, ultimately balancing

tradeoffs among the costs and benefits of weaning to both mothers and infants. However, the particularly large range of flexibility observed in human infant-feeding patterns is a unique aspect of our species' reproduction: wet-nursing, partial or complete bottle-feeding, absence or presence of colostrum-feeding, short periods of breast-feeding, extended periods of breast-feeding, and multi-child breastfeeding (infants and young siblings) are all viable options (Sellen, 2009). Historical, non-historical art sources, and archaeological studies of weaning times using neonatal bone isotope levels all lend support to the diversity of breastfeeding choices women have made in past populations. However, the long-term maternal skeletal health effects of the variation in lactation behaviors are difficult to ascertain in archaeological contexts. Bioarchaeological studies of bone loss in past populations have discussed the potential role of pregnancy and lactation stresses on maternal bone morphology (e.g Agarwal et al., 2004; Agarwal, 2008, 2012; Brickley and Ives, 2008; Agarwal and Grynpas, 1996, 2009; Cho and Stout, 2011; Beauchesne, 2012), but the direct influence of these factors are difficult to ascertain in these contexts when individual reproductive histories are not known. This leaves many questions about the effects of past maternal reproductive choices on skeletal health and bone loss unanswerable. How much time is needed for bone to properly rebuild mineral stores in preparation for the calcium needs of the next offspring? What are the long-term effects of rapid vs. spaced reproductive events on the adult skeleton? In modern contexts, clinicians and epidemiologists studying the effects of reproductive history on female peri and post-menopausal bone health are often confounded by a vast array of biocultural and environmental factors: large differences in maternal diet and nutrition, physical activity patterns, past and present drug use (both illegal and pharmacological), smoking history, and family genetics. Population-specific, socio-cultural factors vary widely within and between human populations and influence the heterogeneity of breastfeeding behaviors (i.e. total duration of lactation, nursing frequency and volume expressed, partial or complete use of formula) (Sellen, 2009), which in turn influence bone metabolism and calcium losses (Prentice et al., 1999; Laskey and Prentice, 1999; Kalkwarf and Specker, 1995; Sowers et al., 2000). Further experimental work is essential in ascertaining the biological understanding needed to better approach both the modern and archaeological records of maternal reproductive behavior and skeletal health.

Animal models have been essential in developing an understanding of the biological and genetic underpinnings of bone metabolism and calcium physiology changes during pregnancy and lactation. Rodent models are the most common animal systems used in bone research, primarily because of lower cost, smaller space requirements, rapid breeding schedule, and short lifespans (McGee-Lawrence et al., 2013). In particular, genetically modified mice (including global as well as bone cell specific knock-ins, knock-outs, and transgenic mice) have greatly enhanced our understanding of bone physiology (e.g. Syed et al., 2010; Syed and Khosa, 2005). Rat studies observing short-term changes to the maternal skeleton during pregnancy and lactation have also been essential, demonstrating large turnover shifts in both cortical and trabecular bone. In cortical bone, large increases in endosteal resorbtion and osteoclastic activity are seen during lactation (Liu et al., 2012; Vadja et al., 2001; Miller and Bowman, 2004), resulting in increased intracortical porosity that does not fully recover even after weaning (Liu et al., 2012). Multiparity has also been associated with larger medullary areas over nulliparous and primiparous controls (Miller and Bowman, 2004). In trabecular bone, lactation is associated with a significant increase in resorbtion over deposition (Toyo et al., 1998), resulting in a significant reduction in plate number, as well as plate-rod and plate-plate junction densities, the

latter of which does not fully recover after weaning (Liu et al., 2012). The post-weaning period is characterized as an anabolic period in which trabecular thickness increases and connectivity improves, though these changes do not result in complete recovery similar to nulliparous control levels (Bowman et al., 2002). Rodent models have also helped to clarify relationships that are difficult to study in humans, including the skeletal ramifications of milk quality on maternal skeletal turnover (O'Dowd et al., 2008; Romano et al.; 2009, 2010), pre-suckling calcium supplementation during lactation (Suntornsaratoon et al., 2014), the effects of exercise on mediating bone loss in pregnant rats (Rosa et al., 2012), and the underpinnings of osteoclastic activity and apoptosis during weaning (Miller and Bowman, 2007). Although helpful in these contexts, there are limits to what rodent models can tell us about human and non-human primate microstructural change and remodeling. The biology of bone metabolism varies from species to species and for rigorous testing of the long-term effects of reproduction on the maternal skeleton, animal models should have similar bone and endocrine changes to those that occur in humans. There are many large differences in rodent vs. human pregnancy endocrinology and skeletal change. Whereas generally a human only delivers and suckles a singleton offspring, the rat gives birth to (and suckles) a large litter of young, the number of which markedly changes the calcium demands during both periods. Approximately 6.7mg/cm^2 of lumbar BMD is lost per rat pup (Toyo et al., 1998). Also in the rat, calcium is uniquely stored in the maternal skeleton during the first half of pregnancy in preparation for the large amount of offspring skeletal mineralization that occurs during the last week of gestation (Ellinger et al.,1952). In rats, maternal bone is not compromised during pregnancy (Halloran and DeLuca, 1980) to the extent that it is in humans (e.g. Janakiraman et al., 2003; Laboissiere et al., 2000; Laskey and Prentice, 1997; Sowers et al., 1995). Rats also lack true skeletal maturity (epiphyseal growth plate closure) and organization of bone into Haversian remodeling (Jee and Yao, 2001; Nelson et al., 1982; Felicio et al., 1984), two essential components of human skeletal aging.

Nonhuman primates (e.g. macaques and baboons) and other large animals (e.g. sheep), are more ideal physiological models of long-term bone change. These species exhibit longer age spans, extended adult skeletal phases, and similar female hormonal cycling patterns (Pope et al., 1989; Jayo et al., 1994; Champ et al., 1996; Zarrinkalam et al., 2012). Large animals are also more appropriate for obtaining bone biopsies for analysis through histomorphometry (Syed et al., 2008). Large population studies investigating bone microstructure variation in captive baboon contexts have helped to clarify the relative effects of biological, environmental, and genetic factors within single species. For example, recent analyses in a large colony of captive baboons (*Papio hamadryas*) have shown clear effects of age, sex, and genetics on several aspects of bone phenotypes (Hansen et al., 2009; Havill et al., 2010), including micro-morphology and remodeling (Havill et al., 2013). And although significant, these factors do not offer a complete explanation of the variation seen at the microstructural level. In particular, a significant percentage of cortical microstructural variation is still left unexplained by age, sex, and genetics in this species (Havill et al., 2013). Clearly other factors, at present unexamined, play important roles in determining bone physiological and functional properties.

Currently very few studies have investigated the effects of parity, lactation, and interbirth interval on determining long-term bone maintenance in large populations of female adult old world monkeys. A large study (n=406) of female captive baboons (*Papio hamadryas*) from the SNPRC found a negative relationship between parity and vertebral aBMD, when all ages were

examined statistically as a whole (Havill et al., 2008). Interestingly, a different study of vertebral BMD in captive rhesus macaques (*Macaca mulatta*) at Cayo Santiago found a positive effect of parity on BMD in young macaques with low parities (<7), and a negative effect in older adults with high parities (>7) (Cerroni et al., 2003). Parity likely has a complicated relationship with age in producing variable bone phenotypes. Cerroni (et al., 2003) noted that in the captive macaque colony, the destructive effects of aging eventually overpowered any beneficial or neutral effect that parity had on BMD earlier in life. Other aspects of reproductive histories (i.e. interbirth interval and lactation durations) have not been examined as thoroughly in these species and may also exhibit influence on bone health parameters. Havill (et al., 2008) found a positive effect of IBI on vertebral aBMD, despite the negative relationship with parity also found in this study. Although it is not clear why IBI had this effect in this population of baboons, longer interbirth interval times likely allow more complete periods of bone replenishment and repair between successive offspring.

Choices of measurement site may also contribute to variation in human and non-human study results as reproduction affects skeletal sites differently, with some sites suffering more significant bone mass losses than others. Cortical and trabecular bone differ from each other structurally, biomechanically, and play different roles in maintaining mineral metabolism and supporting calcium homeostasis. Trabecular (or cancellous) bone forms the inner, spongy region of a bone element and appears to play a larger role in maintaining calcium homeostasis during pregnancy and lactation (Laskey et al., 2011; Glerean et al., 2010; Wisser et al., 2005; Hellmeyer et al., 2006; Pluskiewicz and Drozdzowska, 2002; Matsushita et al., 2002; Honda et al., 1998; Kurl et al., 2002). Cortical (or compact) bone forms the hard outer shell of a bone element and plays a lesser role in mineral homeostasis (Parfitt, 1994a, 2001, 2002a). For example, a study using HSA (hip structural analysis) in women observed differential effects of lactation on cortical and trabecular bone BMDa in the hip region: in particular, increased losses at the trabecular-rich intertrochanter (-3.4%) over the cortical bone-predominant shaft site (-1.7%) (Laskey et al., 2011). The cortical shaft site was also slower to recover after lactation ended, with persisting low BMDa (-1.1%) over the trabecular sites, which showed recovery to postpartum BMDa values by two weeks post-weaning (Laskey et al., 2011). Interestingly, no significant changes to bone width at the shaft were seen, so cortical bone loss occurred at internal surfaces or by increasing intracortical porosity and not at the periosteal surface (Laskey et al., 2011). The loss may have occurred at the endosteum, a mechanically insufficient internal layer that is thickened in women during puberty and acts as a calcium reservoir during pregnancy and lactation (Laskey et al., 2011).

The effects of reproductive history on cortical bone area and remodeling have not been well established in old world monkeys, with most studies focusing only on trabecular-rich sites in the spine and appendicular skeleton (Havill et al., 2008; Cerroni et al., 2003). One study measuring cortical thickness of metacarpals in captive macaques (*Macaca fascicularis* and *M. nemestrina*) found that a history of high parity was associated with relatively thicker bones. Metacarpal thickness was significantly greater for high-parity (>3) females than for low- parity (<3) females in the same age group (Bowden et al., 1979). However, additional work is needed to clarify this relationship and how it may change with advancing age.

Further, while short-term cortical bone remodeling and trabecular architecture changes in old world monkeys have been examined experimentally during the phases of pregnancy, lactation and weaning (Lees and Jerome, 1998; Lees et al., 1998; Ott et al., 1999), the long-term organizing effects over the aging process has not yet been evaluated. This work is essential in advancing our understanding of the cumulative effects of reproductive events on determining microstructural variation throughout aging.

Investigation of bone micro-morphological variation in the adult skeletons of large bodied, captive cercopithecine primates like *Papio hamadryas* from the Southwest National Primate Research Center (SNPRC) with complete individual reproductive histories can offer further insight into how variation in parities, lactation lengths and interbirth intervals affect long-term skeletal micro-organization. Female baboons and humans share many cercopithecine traits, including a large body size, relatively long lifespan, similar reproductive physiology and endocrinology (Brommage, 2001), similar menstrual cycle timing and similar phases of hormonal fluctuations throughout pregnancy and lactation (Chen et al., 1998; Martin et al., 2003). Skeletally, baboons and humans also have the same organization of bone microarchitecture into Haversian systems (Jerome and Peterson, 2001), similar processes of remodeling and repair (Havill et al., 2013), as well as the same patterns of bone degeneration during aging (Aufdemorte et al., 1993; Cerroni et al., 2000; DeRousseau, 1985; Havill et al., 2003a; Kammerer et al., 1995; Havill et al. 2008, 2013). Short-term skeletal modifications during pregnancy and lactation in baboons also parallel those seen in humans, showing progressive bone mass loss during pregnancy through the third trimester (Lees et al., 1998), additional significant bone loss during lactation (Ott et al., 1999), followed by a period of increasing bone mass (recovery) after weaning and resumption of menses (Lees et al., 1998). Captive baboons also exhibit variation in lactation lengths with some females weaning early at six months and others allowing suckling for up to a year (Dr. Linda Brent, personal communication). Female reproductive histories also vary greatly in this captive colony, with females exhibiting a full spectrum of parities, lactation durations, and interbirth intervals. These characteristics make *Papio* an ideal species with which to investigate reproduction-driven variation in micro-organization without the confounding biocultural variables often seen in human studies (e.g. differences in diet, genetics, physical activities, drug use).

Research Objectives

This dissertation is broadly focused on how differences in maternal reproductive behaviors affect cortical and trabecular bone microstructure in an aging experimental baboon model. The literature on both humans and non-human primates indicates pregnancy, lactation, and reproductive history as significant factors in influencing BMD, but little is known about the variation in microstructure that accounts for this change. Modern studies of reproductive history and bone health in aging women are ambiguous and confounded by many biosocial variables. The characterization of skeletal micro-organization differences with reproductive history in a phylogenetically close captive species where many factors (nutrition, exercise) can be controlled will lend considerable clarity into the complicated relationships of bone remodeling changes with

differences in offspring number, lactation history, and average duration between successive offspring.

Building upon the work by Havill et al., (2003a, 2013; Hansen et al., 2009), Chapter 4 explores the relationship of age, sex, and body weight in influencing cortical bone remodeling dynamics and cross-sectional area in male and female captive baboons from the SNPRC. Histological sections of the humerus and femur midshafts were analyzed for differences in secondary remodeling (H.Ar, On.Ar, Dh.Ar, DOn.Ar, Po.Ar, %C/T, %C_A/T) and cross-sectional area (Tt.Ar, Es.Ar, Ct.Ar). This work is important in further establishing that significant differences in appendicular cortical bone dynamics exist by site, and between males and females in this population. This is essential in refuting the null hypothesis (that female reproductive history does not have any bearing on bone microstructure) by demonstrating that males and females exhibit different remodeling patterns.

Because significant differences in bone remodeling and macro morphology do exist between males and females baboons, the remaining chapters focus on exploring the relationship of reproductive history with microstructural changes. Midshaft humerus and femur cortical bone variation in female baboons is further analyzed statistically with reproductive history data in Chapter 5, and female trabecular bone architectural data generated from lumbar micro-CT scans are explored in Chapter 6. Analyzing variation in both structural types of bone (cortical and trabecular) allows for a more complete picture of the complex remodeling changes that occur with reproductive history. In combination with multiple methodologies (histomorphometry and micro-CT) this multi-site approach will also allow a more complete and nuanced examination of bone tissue health and organization across the adult skeleton. As such, the central working hypothesis of this thesis is that reproductive history is a significant factor in generating bone microstructural variation remodeling in both appendicular and axial sites of adult female baboons. A secondary hypothesis is that high offspring production and care, through increased parity and long lactation durations, will have a cumulative effect of increased resorption over deposition and will have negative effects on overall bone health.

This dissertation has four primary goals: 1) to further an understanding of the multifactorial network that influences bone remodeling and microstructure in a primate species closely related to humans; 2) to examine how variation in maternal behaviors affects skeletal morphology at the tissue level across the aging process; 3) to correlate remodeling changes at appendicular sites (humerus, femur) with structural changes at an axial site (L1 vertebra) 4) and to advance anthropological and evolutionary interpretive frameworks through a combined multiple-site and method approach of bone remodeling and microstructure.

Chapter 2

Literature review: Reproduction and mammalian bone
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A. Mammalian iteroparity and the evolution of maternal skeletal resorption and repair

One central evolutionary conflict within mammalian reproduction is the intergenerational transfer of calcium from mother to infant during pregnancy and lactation (Wysolmerski, 2002). Providing necessary amounts of calcium for the rapid growth of the juvenile skeleton during lactation especially presents a heavy metabolic demand on the mother—human mothers must transfer roughly 105g calcium, most of which is harvested from bone mineral even in the presence of a calcium-rich diet (Kovacs, 2001) resulting in typical losses of 5-10% of total maternal bone mass (Kovacs, 2005). Mobilization of calcium requires resorption of previously existing maternal bone tissue, temporarily disrupting the micro-architectural structure, which ultimately provides the basis for withstanding mechanical loading.

Most cercopithecine primates exhibit long periods of offspring care, including extended lactation for their body sizes and late weaning times (Ross, 2001). For example, baboon young suckle for up to two years (Altmann, 1980) and orangutan juveniles for up to 8 years (Galdikas and Wood, 1990). According to evolutionary theory, females in an iteroparous species that produce multiple offspring over time (e.g. monkeys and humans) would have selectively evolved mechanisms to maintain the skeleton over an entire lifetime of reproduction (Ott et al., 1999; Wysolmerski, 2002) to increase reproductive success and optimal offspring survival. If evolution favored mechanisms that conserve and repair skeletal tissues during these reproductive periods, there should be no or little detrimental effect to the skeleton in the long term. One of the adaptations associated with lactation is reversible temporary demineralization of the skeleton (Wysolmerksi, 2010). However the long-term micro-architectural effects of this reversible damage has not been rigorously tested with large samples sizes, multiple methodologies, and multiple bone sites (both cortical and trabecular) within a primate species. The bulk of research investigating the effects of reproduction (pregnancy, lactation, parity, and changes in estrogen) on bone maintenance and micro-architecture has focused on rodents (Jerome and Peterson, 2001). And although small mammals like rats and mice are typically ideal experimental subjects, the large phylogenetic separation from humans, the rapidly spaced successive pregnancies, large-litter reproductive strategy and dramatic disparity in micro-structural organization and metabolism make these data difficult to apply to questions of human and non-human primate skeletal variation. Additionally, non-human primate and human data tend to focus on the short-term alterations to bone microstructure after a single cycle of pregnancy and lactation (e.g. Lees and Jerome, 1998) and not the long-term consequences.

B. Evolution of mammalian lactation, placentation, and calcium-transfer

Lactation:

Lactation is theorized to have evolved during successive radiations of synapsid ancestors, predating the origin of mammals (Oftedal, 2002b). During this period, early apocrine-like glands associated with hair follicles may have provided essential hydration and antimicrobials to thin, parchment-shelled eggs (McClellan et al., 2008; Blackburn et al., 1989; Oftedal, 2002a). Eggs with parchment-like shells are ectohydric and rely on environmental water for completion of egg development (Oftedal, 2002a). It is unlikely that the initial function of milk would have been related to calcium transfer, as there is already a major source of calcium located within the egg: the yolk. The calcium used for egg production comes, in part, from the mobilization of internal stores from bones, scales, and, in turtles, shells (Wysolmerski, 2002). The process of egg yolk formation, or vitellogenesis, involves a period of maternal hyperestrogenism, hypercalcemia, and bone resorption (Schjeide, 1985; Lance et al., 2001) and production of vitelogenin proteins. These proteins have high phosphate contents that bind to circulating calcium (Schjeide, 1985; Feinblatt, 1982) and during oocyte maturation this package of protein, phosphate, and calcium is transported into the yolk for future use by the embryo (Schjeide, 1985). During the later evolution of the therapsids and mammaliaforms, the maternal mechanisms that mobilize calcium for egg production may have served as the template for the evolution of maternal calcium metabolism during mammalian placental transport and milk production (Wysolmerski, 2002). The various components of calcium metabolism during egg production resemble a hybrid of the patterns described for extant pregnancy and lactation in mammals. The mobilization of skeletal calcium stores for egg reproductive purposes predated and was likely the blueprint for the development of mineral metabolism in lactation in mammals (Wysolmerski, 2010).

As the evolution of the importance of egg-yolk production in mammals decreased and the corresponding nutritive content of milk gradually increased, the physiology of milk synthesis and the anatomy of milk delivery evolved in response to environmental and ecological pressures (Akers, 2002; Oftedal, 2002b). The evolution of placenta-based reproduction displaced the initial function of milk as a source of water and nutrients for the egg, leading to the secretion of more calcium-rich, complex milk throughout lactation. Breastmilk from all extant species of mammals examined (including marsupials and monotremes) are rich in calcium (Griffiths, 1968; Green and Merchant, 1988).

Lactation as a strategy for nutrient delivery may have also led to several other important extant mammalian characteristics, including the ability to live in impoverished and unpredictable environments (Pond, 1977, 1984). Particularly, lactation enables mammals to reproduce successfully in environments that support the mother but are otherwise inhospitable to infants (Blackburn et al., 1989; Pond, 1977). Through the synthesis of milk from mammalian stores of fat and minerals, mammalian mothers are not required to continually forage for food to provision their young, thereby buffering mothers and infants from current environmental conditions (Oftedal, 1984; Pond, 1977; Pond, 1984). And although the energetic costs of milk synthesis versus provisioning solid foods are often equal for the mother, milk is typically more digestible in infants than are solid foods that require mastication and detoxification. Additionally, there are

behavioral and psychological aspects of suckling and nurturing between mother and offspring that produce bonds that promote neonate survival. This is an important aspect of lactation that exists along with the chemical and physical characteristics of milk and also lends to offspring growth and development.

Placentation:

As the function of lactation became the primary mammalian adaptation for transfer of maternal resources to the offspring postnatally, this enabled a reduction of maternal resources directed into the egg. The role of the yolk decreased over time in the mammaliaform evolution. Ancestral egg-laying (ovipary) as a strategy is retained in the extant monotremes, but lost in favor of viviparity in marsupials (Metatheria) and placental mammals (Eutheria). Viviparity (live-born young) with placentation is not a unique feature of mammals and has evolved more than 100 times within the different lineages of non-mammalian amniotes, especially in lizards and snakes (Lombardi and Wourms, 1985; Blackburn, 1993; Hamlet, 1989; Mess et al., 2003; Stewart et al., 2006; Crespi and Semeniuk, 2004). Placentation in general refers to all conditions under which adjacent and integrated embryonic and maternal tissues accomplish physiological exchange in viviparous species (e.g., Mossman, 1937, 1987; Starck, 1959). This physiological exchange can include nutrients (including calcium), gas exchange, and transfer of wastes.

Although ovipary is retained in monotremes, to a large extent embryonic development still takes place internally. Platypus eggs develop in utero for approximately 28 days before they are laid. The mother platypus then incubates them with her body heat for an additional 10 days before they hatch. This egg-laying strategy is in contrast with birds (i.e. chickens) in which the egg develops for only 24 hours within the maternal body before being laid and is then incubated externally for 20 or more days until the young hatch. The extended in-utero development in monotremes is facilitated by a chorio-vitteline or yolk-sac placenta. The chorio-vitteline placenta is shared by both monotremes and marsupials and is likely an ancestral trait. In placental mammals, a rudimentary yolk-sac placenta forms first but then is replaced by a more derived chorio-allantoic placenta for the remainder of the pregnancy.

 In all placental mammals, the calcium transfer from the mother to fetus occurs via an active mechanism (Smith and Moe, 1992) based on two observations: first, that calcium concentration is higher in the fetus than in maternal blood and, second, the perfusion in situ of the umbilical artery resulted in a net increase of the calcium concentration of the perfusate in the fetus (reviewed in Tivane et al., 2013). Active calcium transport is also facilitated by a specialized region of the placenta called the syncytiotrophoblast (Brunette, 1988). The syncytiotrophoblast transports approximately 80% of calcium from maternal to fetal circulation (Marin et al., 2008) and is a major component facilitating in-utero calcium transfer.

C. Calcium economy during pregnancy

During pregnancy, calcium is transferred across the placenta primarily for fetal skeletal mineralization. At birth, a human neonate has accumulated approximately 30g of calcium and has formed 98% of its skeleton. Active transport of calcium across the placenta begins at week

12 of gestation and peaks at week 36 (Forbes, 1976). Placental calcium transport is dependent upon transport proteins located in the syncytiotrophoblast, which forms a barrier between the mother and fetus (Brunette, 1988). The bulk of this transfer occurs during the third trimester, when approximately 250-350mg of calcium is accumulated by the fetus each day (Kovacs, 1997; Prentice, 2003; Ryan et al., 1988).

Several maternal metabolic and physiological changes help facilitate the increase in maternal absorption, retention, and transfer of calcium to the fetus during pregnancy (**Fig 1**). Cacitropic hormones play a large role in facilitating this. Calcitrol ($1,25(OH)_2D$) increases progressively throughout pregnancy (Ritchie et al., 1998 Cross et al., 1995), as well as parathyroid hormone receptor protein (PTHrP). PTHrP is produced by mammary and fetal tissues primarily to stimulate placental calcium transport to the fetus as well as to increase maternal calcium absorption in the small intestine and tubular resorption in the kidney (Kovacs and Kronenberg, 1997). Both the active ($1,25(OH)_2D$) and inactive ($25(OH)D$) forms of vitamin D help facilitate calcium changes, with the active form increasing twofold during pregnancy, allowing intestinal absorption of calcium to also double (Ritchie et al., 1998, Zeni et al., 2003). The inactive form of vitamin D appears to play a role in fetal bone development, but this is not well understood (Dror and Allen, 2010). Insulin growth factor (IGF-1) stimulates maternal bone turnover during pregnancy, particularly bone resorption (O'Brien et al., 2006). In the third trimester, IGF-1, calcitrol, and calcium intake explain 88% of the variability in net bone maternal calcium balance (O'Brien et al., 2006).

Non-invasive bone mineral density (BMD) changes at maternal skeletal sites during human pregnancy and lactation are typically evaluated through one of four methods: dual-energy x-ray absorptiometry (DEXA), quantitative computed tomography (QCT), peripheral QCT (pQCT), and quantitative ultrasound (QUS). No one method is perfect in evaluating mineral changes to bone sites (see review in Olausson et al., 2012). Trabecular-rich sites like the hip, proximal femur, distal femur, distal radius, and lumbar spine are typically chosen for analysis. Biochemical markers of bone turnover can also be evaluated via maternal blood sample by measuring collagen breakdown products (e.g. deoxypyridinoline), hydroxyproline and C-telopeptide (CTX) to gain an understanding of the relative changes in bone resorption and deposition throughout the three trimesters. A review of the present data is presented further on in this chapter.

D. Calcium economy during lactation

In contrast to the maternal hyper-estrogenic period of pregnancy, lactation is characterized by low circulating estrogen concentrations, caused by prolactin-driven amenorrhea. Several researchers have postulated that the increased bone turnover and net changes in bone mineral during lactation may be related to this low estrogen state (Kolthoff et al., 1998; Kalkwarf et al., 1997; Affinito et al., 1996; Krebs et al., 1997). However, Kovacs and Kronenberg (1997) have emphasized that the rate of bone loss in nursing women far exceeds that in women rendered estrogen deficient with GnRH analogues so low estrogen alone is unlikely to drive the fast and significant bone loss seen during breastfeeding. Additionally, the many calcium-retaining mechanisms seen during pregnancy are not continued during lactation, causing

an increased dependence upon skeletal mineral sources. By 2-3 months postpartum in both breastfeeding and nonbreastfeeding mothers, intestinal calcium absorption values return to those seen during pre-pregnancy or early gestation (Ritchie et al., 1998; Vargas et al., 2004; Cross et al., 1995; Kalkwarf et al., 1996), though there is some evidence that fractional absorption is higher in breastfeeding women who have resumed menses than those who are still experiencing amenorrhea at the same time point postpartum (Kalkwarf et al., 1996). Glomerular filtration rates are reduced after parturition (Kovacs, 2005) and urinary calcium excretion also returns to levels seen in pre-pregnancy or in non-pregnant, non-breastfeeding women (Naylor et al., 2000; Prentice, 2000). Postpartum, both plasma volume and total plasma calcium levels return to pre-pregnancy values (Black et al., 2000; Ritchie et al., 1998; Kent et al., 1990; Kovacs, 2005). Breastfeeding women have total and ionized plasma calcium levels similar to those seen in non-breastfeeding women at the same time postpartum (Prentice, 2003; Kalkwarf et al., 1999). During the first few months postpartum, increases in $1,25(OH)_2D$ have been reported in both breastfeeding women and non-breastfeeding women (Krebs et al., 1997) and early postpartum changes to PTH and $1,25(OH)_2D$ do not seem correlate with breastmilk calcium levels, bone turnover markers, or bone mineral changes (Ritchie et al., 1998; Krebs et al., 1997; Sowers et al., 1998). However, specifically in late lactation and early weaning, elevated PTH and $1,25(OH)_2D$ is seen in breastfeeding women and not in other groups (i.e. in early lactation or non-breastfeeding groups) (Kalkwarf et al., 1997; Kalkwarf et al., 1999; More et al., 2003; Prentice et al., 1998; Kent et al., 1990; Cross et al., 1995; Specker et al., 1991) and may play a more significant role during this time. During late lactation only, elevated PTH and $1,25(OH)_2D$ together stimulate increased intestinal absorption and renal retention of calcium, possibly leading to decreased need for calcium from the skeleton and during this phase of breastfeeding.

After breastmilk transitions from the early colostrum, calcium concentrations are relatively constant during the first 3 months of lactation, averaging about 200-300mg/l, depending on the population (Prentice et al., 1999), and this amount progressively declines thereafter (Prentice et al., 1999; Vaughan et al., 1979; Laskey et al., 1990). Studies have shown large differences in breastmilk calcium levels between individual mothers as well as between populations at the same time point postpartum (Prentice et al., 1999; Jarjou et al., 2012). Calcium is associated with the casein, phosphate and citrate components of human breast milk, and regulation of these fractions likely play a role in determining total breastmilk mineral concentrations (Kent et al., 2009). There is also some evidence that polymorphisms in PTH/PTH-related protein (PTHrP) receptor 1 gene are associated with variation in breastmilk calcium concentration (Jones et al., 2008). PTHrP is produced by the lactating mammary gland, in association with prolactin, and is released into the maternal bloodstream and into breastmilk (Lippuner et al., 1996; Sowers et al., 1996). Plasma concentration of PTHrP is highest after delivery and declines thereafter (Dobnig et al., 1995; Sowers et al., 1996). In the few weeks postpartum, increased PTHrP levels are seen in breastfeeding women as compared to non-breastfeeding women (Sowers et al., 1996), but is virtually undetectable at 6 months postpartum even if breastfeeding is continued (Sowers et al., 1996, Grill et al., 1992).

E. Short-term changes to bone mineral during and after pregnancy and lactation

Humans

The skeleton of an adult woman contains approximately 1kg of calcium (Widdowson and Dickerson, 1964). A meta-data analysis by Olausson et al., (2012) found that post-partum whole body BMC measurements range from a significant decrease of -2.0% to a non-significant change of +0.5%. The -2.0% decrease in whole-body bone mineral equates to approximately 25g calcium lost, the amount necessary for total fetal bone accretion (Olausson et al., 2008).   Site-specific changes to bone mineral during pregnancy also vary by sample population. For example, for the distal radius alone, some studies have found significant changes in bone mineral from mid-pregnancy to shorty after birth (Kolthoff et al., 1998; More et al., 2001), whereas other studies have not found significant changes during this period (Black et al., 2000; Kent et al., 1993). A meta-data analysis (Olausson et al., 2012) found mean changes during pregnancy ranging from -4.5 to -0.9% at the lumbar spine, -3.6 to +1.8% at total hip, -4.8 to -1.2% at the femoral trochanter, -2.4 to +1.2% at the femoral neck, -3.8 to +1.3% at the radial shaft and -3.8 to +1.3% at the radius. Losses at single sites appear to be vary depending on if trabecular or cortical bone is assessed; for example, one study using pQCT (Wisser et al., 2005) found significant decreases in vBMD between the first and third trimester of pregnancy in the trabecular but not cortical region of the bone. Bone loss will also vary by individual depending on pre-pregnancy conditions, nutrition, age, and genetics (Prentice, 2003). Furthermore, low or high BMI (Sowers et al., 1991; Butte et al., 2003), significant weight gain during pregnancy (Olausson et al., 2008), advancing age (Olausson et al., 2008), calcium supplementation ≥1000mg/day (Janakiraman et al., 2003; Laboissiere et al., 2000), and pregnancy immediately following a period of extended lactation (Laskey and Prentice, 1997; Sowers et al., 1995) have all been shown to significantly alter site-specific patterns of bone mineral losses during pregnancy.

Pregnancy-specific changes to bone remodeling and micro-architecture outside of BMD are difficult to study in human women. Bone biopsies offer the most specific information about micro-architectural and remodeling changes but are not preformed because the surgery and anesthesia required puts both the mother and fetus at an unnecessary risk. However, there is one study that took one set of iliac biopsies from women during early pregnancy (8–10 weeks) at the time of elective abortion, a second set from women in late pregnancy (39–40 weeks) at the time of elective c-section, and a third set from healthy non-pregnant, non-lactating women (Purdie et al., 1988), thereby eliminating unnecessary surgical risks. This study showed decreased bone volume, decreased bone deposition and increased bone resorption in the early pregnancy iliac biopsies, when compared with the nonpregnant cohort. The biopsies from late pregnancy showed increased bone deposition over resorption, and the bone volume was equivalent to that seen in the nonpregnant group. A second researcher utilized the same biopsies to study the specific changes to trabecular organization (Shahtaheri et al., 1999). This study found that a notable feature of early pregnancy was a decline in the trabecular thickness and a reduction in trabecular nodes (junctions). The node: terminus ratio (the index of trabecular discontinuity) was lower in early pregnancy but repaired in late pregnancy, suggesting that the initial disconnection of the trabecular network was partially reconnected by term (Shahtaheri et al., 1999). However

because the three sets of biopsies in Shahtaheri (et al., 1999) are from disparate groups of women, and parity was not matched in the late-pregnancy and non-pregnant groups, it is difficult to say if the remodeling and trabecular architecture patterns seen in this study are truly characteristic.

Biochemical markers taken from maternal blood and urine samples offer another indirect, non-invasive view of remodeling. Markers of both bone formation and resorption increase significantly from the first to third trimesters (Zeni et al., 2003; Bezerra et al., 2004). The resorption markers carboxyl terminal collagen cross-links (CTX), n-telopetide cross-links (NTX), and deoxypyridinoline have all been detected as early as the first trimester (Ulrich et al., 2003; Naylor et al., 2000; Black et al., 2000), with CTX and NTX showing the largest increases between the second and third trimesters (Zeni et al. 2003; Naylor et al. 2003). Two markers of bone formation, pro-collagen type-1 carboxylterminal propeptide and bone-specific alkaline phosphatase, also show low levels earlier in pregnancy (Ulrich et al., 2003; Naylor et al., 2000, Black et al., 2000; Kaur et al., 2003) but exhibit a 44% increase between the second and third trimesters (Zeni et al., 2003). These data indicate that although the maternal skeleton is actively resorbing bone to increase circulating calcium levels during the later stages of pregnancy, some deposition is also occurring to balance this process, possibly explaining the equivalent bone volume data seen in late pregnancy and non-pregnant women in previous studies (Purdie et al., (1988; Shahtaheri et al., 1999). However in general, data from biomarkers during pregnancy must be interpreted with some caution as there are several factors that directly influence biomarker ratios and absolute values, including intra-individual variation in response to changes to circadian rhythms and recent digestion of food (Naylor et al., 2000). Blood-borne analytics are also influenced by increases in plasma volume, haemodilution, and increased glomerular filtration rates, all of which are typical during pregnancy (Naylor et al., 2000).

Lactation is also a period characterized by high calcium mobilization and bone turnover, and in most cases, more so over pregnancy. Biochemical markers of turnover show higher concentrations during the first five weeks postpartum than in non-pregnant, non-lactating women (Yasumizu et al., 1998; Paoletti et al., 2003; Kent et al., 1990; Casanueva et al., 2004). Typically, bone mineral mobilization occurs when resorption exceeds formation, and skeletal replenishment occurs when formation exceeds resorption. However, significant bone loss is observed in lactating women despite high biomarkers of both deposition and resorption (Sowers et al., 1995; Carneiro et al., 2010). This is puzzling since the coupled action of osteoblasts and osteoclasts should theoretically yield no net changes to bone mineral density. However, previous work has shown that increased PTHrP in combination with suppressed estrogen levels causes an increase in osteoclast-mediated bone resorption (Kovacs, 2005; Van Houten and Wysolmerski, 2003; Burtis et al., 1990). In the rat model, continuously elevated PTHrP also recruits osteoblast precursors and initiates the osteoblastic differentiation program, but complete cellular differentiation and mineralization does not occur (Dobnig and Turner, 1997; van der Horst et al., 2005). This mechanism of increased osteoblast recruitment with incomplete cellular differentiation explains the conundrum of elevated bone formation markers and increased bone loss seen in breastfeeding women (Sowers et al., 1995; Carneiro et al., 2010).

Longitudinal and observational studies have established that BMD declines approximately 5-10% during lactation (Kovacs, 2005), with greater losses from trabecular than

cortical bone and greater losses from axial than appendicular sites (Laskey et al., 2011; Glerean et al., 2010; Hellmeyer et al., 2006; Chan et al., 2005; Pluskiewicz and Drozdzowska, 2002; Matsushita et al., 2002; Honda et al., 1998; Kurl et al., 2002). Mean changes to maternal bone mineral in breastfeeding women range from -7.5 to -2.8% at the lumbar spine, -4.2 to -1.5% at the total hip, -5.0 to +0.3% at the radial wrist and -0.1 to +0.6 at the radial shaft (Prentice et al., 1999; Laskey and Prentice, 1999; Kolthoff et al., 1998; More et al., 2001; Pearson et al., 2004; Ritchie et al., 1998; Akesson et al., 2004; Karlsson et al., 2001; Sowers et al., 1993). Similar patterns of bone loss are not observed in non-breastfeeding women postpartum. In postpartum measurements of Caucasian non-breastfeeding women, no net losses were observed at the wrist (More et al., 2001), and trochanter (Pearson et al., 2004), whereas significant losses were seen at these site in women who breastfed between 3-12 months. Women who breastfeed also show higher concentrations of bone turnover makers postpartum than mothers who do not breastfeed (Yamaga et al., 1996; Chan et al., 2005; Kalkwarf et al., 1999; Casanueva et al., 2004; Sowers et al., 1995).

The severity of lactation-driven bone loss at different sites varies by population. Although Caucasian women typically lose 7.5 to 2.8% bone at the lumbar spine, one study of Chinese women who exclusively breastfed for at least 3 months showed only -1% aBMD at the spine as compared to measurements at one week postpartum (Chan et al., 2005). However, considerable variation in bone response is seen even among women who lactate for the same length of time within the same population. Cambridge, UK mothers who breastfed exclusively for 3 months varied from -8.5 to +1.2% in BA-adjust BMC at the lumbar spine (Laskey et al., 1998). In this sample, maternal height and breast milk volume were identified as significant factors influencing variation in bone response (Laskey et al., 1998), though there are likely many other factors at play. Differences in breast-feeding practices, such as the intensity and frequency of feeding, the volume of breast milk produced and the timing of the introduction of complementary foods may explain much of the variation seen in bone mineral changes during lactation (Prentice et al., 1999; Laskey et al., 1998). For example, women nursing twins and triplets lose significantly more bone than women nursing a singleton (Laskey et al., 1998; Peng et al., 1988), indicating a strong effect of total milk volume on determining bone loss.

Total length of time breastfeeding has also been shown to have strong effects on maternal bone volume. During a 3-6 month postpartum period, longer breastfeeding durations are associated with larger decreases in bone mineral than are shorter durations of breast-feeding (Laskey and Prentice, 1999; Hopkinson et al., 2000; Sowers et al., 1993, Yasumizu et al., 1998). Studies of bone mineral losses seen in very extended lactation (18+ months) are rare but studies of Gambian women, who typically breastfeed 18-24 months with long periods of lactation-driven amenorrhea, show only partial reversal of skeletal change by 12 months postpartum (Jarjou 2004). However these same Gambian women showed significantly higher whole body, lumbar spine, and hip BMC and aBMD after completion of weaning and resumption of menses as compared to the scans taken at 12 months lactation (Jarjou et al., 2013). This indicates that full recovery of bone mass is possible even after extensive breastfeeding and a low-calcium (<400 mg/day) diet (Sawo et al., 2013).

As in pregnancy, trabecular bone appears to be preferentially targeted and net changes of -4% vBMD in the trabeculae at the distal radius was observed by 6 months of lactation (Dobnig et al., 1995). However in contrast to pregnancy, BMI and postpartum weight-changes are not significantly associated with change in BMC, aBMD and BA-adjusted BMC during lactation (Laskey and Prentice, 1999; Laskey et al., 1998), or are only minimally influential (Kolthoff et al., 1998; Karlsson et al., 2001; Hopkinson et al., 2000; Polatti et al,. 1999; Kalkwarf et al., 1999; Kalkwarf, 1999). Lactation-specific losses are seen in the lumbar spine and femoral neck in multiple populations of women, including Caucasian, Japanese, Chinese, and Chilean (Sowers et al., 1993; Yasumizu et al., 1998; Chan et al., 2005; Lopez et al,. 1996).

Resumption of menses seems to play a large role in moderating skeletal repair. One study showed that both the duration of exclusive breastfeeding and length of postpartum amenorrhea were positively associated with high levels of both bone formation and resorption markers (Holmberg-Marttila et al., 2003). In general, the large bone mineral losses seen in early lactation are typically reversed upon resumption of menses (Kalkwarf and Specker, 1995; Kolthoff et al., 1998; Lopez et al., 1996; Sowers et al., 1993). There are many reasons why this may be, including reduction of breastfeeding bouts and duration, and significant hormonal changes as normal monthly cycling begins (Olausson et al., 2012). Additional and interrelated factors include daily milk output, plasma estrodial levels and overall length of amenorrhea, all of which have been predictive of bone loss when considered separately but not together (Prentice et al,. 1999; Laskey and Prentice, 1999; Kalkwarf and Specker, 1995; Sowers et al., 2000). In one study using QCT, no net changes were observed in the trabeculae of the spine 5 months after the resumption of menses (compared with scans taken before pregnancy), even though some of the women in the study were still breastfeeding (Ritchie et al., 1998). Resumption of regular cycling and the subsequent increase in circulating estrogen levels may be the single most important factor in the reversal of lactation-specific bone loss (Carneiro et al., 2010, Sawo et al., 2013). This may be because complete osteoblast and osteoclast differentiation does not occur until after lactation stops (Carneiro et al., 2010).

Old world monkeys

Several studies have demonstrated that old world monkeys (i.e. macaques and baboons) undergo similar physiological and metabolic changes during pregnancy and lactation, as compared to human women. For example, both macaques and baboons exhibit estrogen and progesterone ratios during pregnancy similar to those seen in women, with extremely elevated levels of estrogen during pregnancy and abrupt decreases in estrogen and progesterone immediately postpartum (*macaques* Hein et al., 1989; *baboons* Hendrickx and Dukelow, 1995). Lactational amenorrhea lasts for 4–6 months postpartum in breastfeeding macaques (Weiss et al., 1976), and infants acquire approximately 7.5–10 g calcium during pregnancy and lactation, which equals approximately 5.0–6.6% of the maternal calcium stores (Lees et al., 1998).

During pregnancy no obvious changes in bone mass, remodeling biomarkers or histological parameters were seen in cynomolgus monkeys (Lees and Jerome, 1998; Lees et al., 1998), pig-tailed macaques (Ott et al., 1999), vervet monkeys (Hiyaoka et al,. 1996) and marmosets (Power et al., 1999). Researchers concluded that the high serum estrogen levels

allowed for the balanced coupling of remodeling and modeling during pregnancy in these species. Large calcium supplementation in monkey chow may also play a role (Lees and Jerome, 1998). In two studies, invasive procedures were used to obtain iliac bone biopsies both during pregnancy and at postpartum and little change was seen in the micro-cellular components as compared to pre-pregnancy samples (e.g. Ott et al., 1999; Lees and Jerome, 1998) so remodeling and subsequent bone changes do not seem to be significant during this period. However, it is important to note that these studies typically use young adult animals who are skeletally mature but may not have reached peak bone mass yet. Short-term pregnancy-driven metabolic changes may be markedly different in animals who have already reached peak bone mass.

As in humans, non-human primate data indicates the lactation period as significant in driving maternal bone mineral losses (e.g. Lees et al., 1998; Ott et al., 1999). In macaques, the maternal losses seen during lactation (approx. 99 mg/day) are reflected in the gains seen in the offspring (approx. 78 mg/day, between 2– 4 months of age). These findings support the human studies that indicate that the skeleton often serves as the primary source of calcium during lactation (Kent et al,. 1993). During lactation, macaques lose large amounts of bone mass even in the presence of a high-calcium diet (monkey supplementation is 160 mg/kg, or approximately 8x the recommended intake for women) (Lees et al., 1998). This is similar to the observation seen in Prentice et al., (1995): the bone mass of Gambian women who received calcium supplementation (up to 714 mg/day) was not significantly different from that of non-supplemented Gambian women during lactation. These data suggest that underlying mechanisms, possibly including decreased estrogen concentrations, causes the skeleton, rather than dietary sources, to serve as one of the main mineral resources during lactation (Lees et al., 1998; Kent et al., 1993; Prentice et al., 1995). In macaques, an observed increased turnover rate had not yet abated by 9 months postpartum in macaques (Lees and Jerome, 1998), and BMD measurements had not returned to pre-pregnancy values by 10 months postpartum in both macaques and African green monkeys (Lees et al., 1998, Hiyaoka et al,. 1996), indicating that postpartum bone recovery in these species is slow and lengthy. One study observed complete restoration of bone mass by three months in pig-tailed macaques (Ott et al., 1999), but the monkeys used in this study were again very young macaques (approx. 3 years old, i.e. a model of human adolescent pregnancy) so the quick recovery of bone is more likely related to the continuation of the growth process and not lactation itself.

F. Long-term changes to BMD and susceptibility to fracture in old age

Parity and Lactation History in Humans

There is some literature supporting a positive association between parity and improved bone health in pre-and post-menopausal women (e.g. Hillier et al., 2003; Cure-Cure et al., 2002). Potentially there could be an adaptive mechanism in women with multiple pregnancies to protect against increased bone loss after the first pregnancy. There are some data to support this theory. For example, greater bone mass losses during pregnancy have been reported in primiparous women than in multiparous women (Sowers et al., 2000). And one marker of bone resorption,

hydroxyproline, is increased 58% more in primiparous than in multiparous women (Donangelo et al., 1996). These data point to an increased rate of bone resorption during first time pregnancy as compared to subsequent pregnancies. Reduced resorptive remodeling in successive pregnancies could lead to neutral or positive outcomes on BMD later in life. A cohort study assessing 9,704 women over the age of 65 years showed that nulliparous women had a 44% increased risk of hip fracture as compared with parous women, when adjusted for BMD and BMI (Hillier et al., 2003). When assessed by parity group, the probability of hip fracture decreased as parity increased. Three additional studies have reported a similar pattern of reduced hip fracture rate with increased parity (Hillier et al., 2003; Michaelsson et al., 2001; Cure-Cure et al., 2002). High parity has also been shown to have a protective effect against menopausal bone loss and development of osteoporosis in some populations (Lenora et al., 2009; Karlsson et al., 2005; Fox et al., 1993; Cure-Cure et al., 2002; Okyay et al., 2013). There are several possibilities as to why high parity may have protective influences on BMD and fracture risk: a cumulative effect of increased circulating estrogen levels during pregnancies, periods of weight gain and subsequent increased biomechanical loading, increased dietary calcium through prenatal vitamin consumption, and increased daily physical activity associated with child-rearing (reviewed in Olausson et al., 2012). Despite these possible protective effects, several epidemiological studies have shown a neutral effect of parity on bone health in old age. Two retrospective studies found no association of aBMD with parity in Bangladeshi or Sri Lankan women (Chowdhury et al,. 2002; Lenora et al., 2009). Another found no difference in bone measurements between South African Bantu women who had given birth to two or fewer children compared with those who had birthed seven or more (Walker et al., 1972). Other reports show no significant association between parity and BMD or development of osteoporosis (Ensom et al., 2002; Melton et al., 1993; Henderson et al., 2000; Alderman et al., 1986; Kojima et al., 2002; Bererhi et al., 1996; Kritz-Silverstein et al., 1992). The Finnish-American women studied in Henderson et al., (2000) are characterized by multiple, closely spaced pregnancies, and allowing time for recovery between subsequent pregnancies may be an important aspect to conferring a protective effect later in life. Although rare, two retrospective studies have observed a negative effect of multiparity on bone density (Allali et al., 2007; Gur et al., 2003). In Gur et al., (2003), Turkish pre and post-menopausal women with parities over 5 had significantly lower BMD at the trochanter and lumbar spine, as compared to women with 1-2 parities. Lower BMD values with high parity were seen across several pre and post-menopausal age groups, with many other social factors kept constant (calcium, alcohol, and coffee intake, physical activity, etc). Similar results were seen in a study of Moroccan women, showing decreased lumbar and hip BMD with high parity (<6 births) (Allali et al., 2007). The authors suggest that low calcium intake, extended breastfeeding, and low-vitamin D exposure as a result of full-body veils, may explain the negative association seen in this population.

Retrospective studies of lactational history on long-term (i.e. pre and post-menopausal) bone health are mixed. Spanish pre-menopausal women with a lactational history had higher BMD at the lumbar spine and higher aBMD, both total and cortical, than women who did not breastfeed (Canal-Macias et al., 2013), and another study found a beneficial effect of breastfeeding on delaying osteoporosis (Schnatz et al., 2010). Some retrospective studies have also demonstrated a strong positive relationship between lactation history and reduced hip fracture rate in old age (Alderman et al., 1986; Kreiger et al., 1982; Kreiger et al., 1992; Cumming and Klineberg, 1993). However other studies have found no significant relationship

between breastfeeding and BMD or osteoporosis risk (Chowdhury et al., 2002; Lenora et al., 2009; Kojima et al., 2002). The variation in long-term bone response to lactation is likely related to the heterogeneity of lactation behaviors across study populations, including differences in the timing, duration and intensity of breastfeeding. As reviewed earlier in this chapter, the data on short-term changes to BMD during lactation all point to variations in these behaviors as significant in affecting overall bone loss and metabolism (Prentice et al., 1999; Laskey et al., 1998), and these may have lasting effects. Exclusive breastfeeding, as opposed to breastfeeding supplemented with formula or complementary foods, may affect bone loss in a different way than other methods. Pearce (2006) found that a U.S. cohort of women who lactated more intensively (i.e. exclusive infant breastfeeding only) had greater bone density values than those women who did not. Total duration of breastfeeding per child may also be a significant factor. There is some evidence that histories of extended breastfeeding (longer than one year) per child in combination with high parity may be associated with low pre and post-menopausal BMD, especially in women who experience low-calcium intake, low dietary nutrition, and low socioeconomic status (Henderson et al., 2000; Dursun et al., 2006; Demir et al., 2008; Hopkinson et al, 2000; Okyay et al., 2013). A significant negative correlation was seen between extended breastfeeding histories of 24-36 months and BMD in Rojano-Mejia et al., (2011), and a similar negative relationship was observed in breastfeeding past one year in Okyay et al., (2013). Extended periods of lactation may impair the natural process of bone recovery. In More et al., (2001), recovery of bone loss was seen in all participants except those who breastfed for a longer (1 year) period of time. In Turkish women, a history of prolonged breastfeeding of 1 year or longer was associated with an increased risk of osteoporosis (Okyay et al., 2013). Interestingly, in Amazonia Shuar women, extended lactation had a protective effect on BMD in women aged 35-44 years old, but not in the peri and post menopausal age groups (Madimenos et al., 2012). In all the studies, any protective effect of extensive breastfeeding appears to disappear by menopause.

A woman's overall lifetime reproductive pattern of age of first menarche, age at first parturition, total parity, average interbirth interval, total number of menstrual cycles, and duration of lactational-driven amenorrhea may also play a role in how reproductive history strengthens or weakens bone prior to menopause. There are some population-specific patterns to consider as well. Women in industrialized Western countries tend to experience early menarche, later age at first birth, low total parity, short lactation periods, and overall larger total number of lifetime menstrual cycles (Eaton et al., 1994; Strassman, 1997; Whitten, 2008). In contrast, women in non-industrialized countries often experience later menarche, earlier first parturition, greater total parities, on average 3-4 years of lactation per child, and as a result, overall fewer menstrual cycles over the lifetime (Sperling and Beyene, 1997; Weaver, 1998). These different overall patterns are accompanied by large alterations in the relative amounts of circulating sex hormones throughout adulthood, which will have strong effects on bone health and BMD. Though the relative importance of each of the components in these different patterns is difficult to ascertain, but there are some trends emerging. For example, some researchers have suggested that earlier age at first menarche, and the corresponding increase in estrogen levels (with the achievement of monthly cycling) will have a stimulating effect on bone deposition by increasing osteoblastic activity earlier and establishing a higher peak bone mass, potentially leading to an increased BMD by the time of menopause (Ito et al., 1995; Jaffe and Dell'Acqua, 1985; Roy et al., 2003). Some studies of postmenopausal BMD measurements have found evidence of a

positive relationship between earlier menarche and increased menopausal BMD (Gerdhem and Obrant, 2004; Roy et al., 2003; Silman, 2003) whereas other studies have not (Ito et al., 1995; Ozdemir et al., 2005; Sioka et al., 2010; Varenna et al., 1999). One study of skeletal health in Amazonia Shuar women, a natural fertility and subsistence population in Ecuador, found a positive relationship between earlier menarche and BMD in postmenopausal women, but not in pre-menopausal women (Madimenos et al,. 2012), so the effects of early vs. late menarche may not be seen until the losses associated with menopause have begun. An interrelated variable, later age at first pregnancy, may also have a significant effect on bone health later in life. Theoretically, a woman's BMD continues to increase and peak throughout her twenties (Abrams, 2003) and disruptions to this important process of bone formation may cause decreased peak bone mass accrual. Although one study observed no effect of early first pregnancy on bone mass accrual (Sowers et al., 1985), several studies have demonstrated a negative effect of early first pregnancy on BMD in old age (Hayslip et al., 1989; Kent et al., 1990; Schnatz et al., 2010; Sowers et al., 1993).

Baboons and Macaques

Currently very few studies have investigated the effects of parity, lactation, and interbirth interval (IBI) on determining long-term bone maintenance in large populations of female adult old world monkeys.   In rhesus macaques from Cayo Santiago (*Macaca mulatta*) Cerroni et al., (2003) found a positive relationship between vertebral BMD and parity for females aged 4-12.5 years. In a second age range of 9-22.2 years, BMD increased with parity, up to a maximum of 7 offspring. After 7 offspring, BMD declined with increasing parity. The author attributed the positive relationship between BMD and parity to pregnancy-associated weight gain, increased estrogen levels, and increased calcium absorption. The later negative relationship (parity>7) was attributed to the detrimental effects of aging overpowering any possible protective effect of reproduction (Cerroni et al., 2003). A similarly positive relationship between parity and bone health is seen in Bowden et al. (1979). This radiographic study in captive macaques (*Macaca fascicularis and M. nemestrina*) found that high parity is associated with relatively thicker metacarpal bones. The metacarpal cortical area was greater for high-parity (>3 offspring) females than for low-parity (<3 offspring) females in the same age group (Bowden et al., 1979).

In a large study (n=406) of female captive baboons (*Papio hamadryas*) Havill et al., (2008) found a mean negative relationship between parity and vertebral aBMD, but a positive relationship was seen between IBI and aBMD at the same site. No relationship between reproductive variables and BMD was seen at the distal radius, even though both the distal radius and vertebral sites both contain a relative large amount of trabecular bone. The distal radius is utilized in quadrupedal locomotion in this species and the significant biomechanical loading may offset any reproductive changes. The negative relationship between parity and BMD seen in Havill et al., (2008) likely differs from the positive relationship seen in Cerroni et al,. (2003) because the former analyzed the relationship as a whole over all ages and parities whereas the latter analyzed the relationship by specific age groups and parities. It is possible that the relationship of parity to BMD in populations will differ within specific age cohorts (i.e. as seen in Cerroni et al., 2003) and the overall effects of parity will be skewed if the entire spectrum of ages is analyzed statistically as a single group. Additionally, the positive relationship of IBI and

vertebral BMD in Havill et al., (2008) further highlights the complexity of interpreting the various effects of pregnancy and lactation on bone health over multiple skeletal sites.
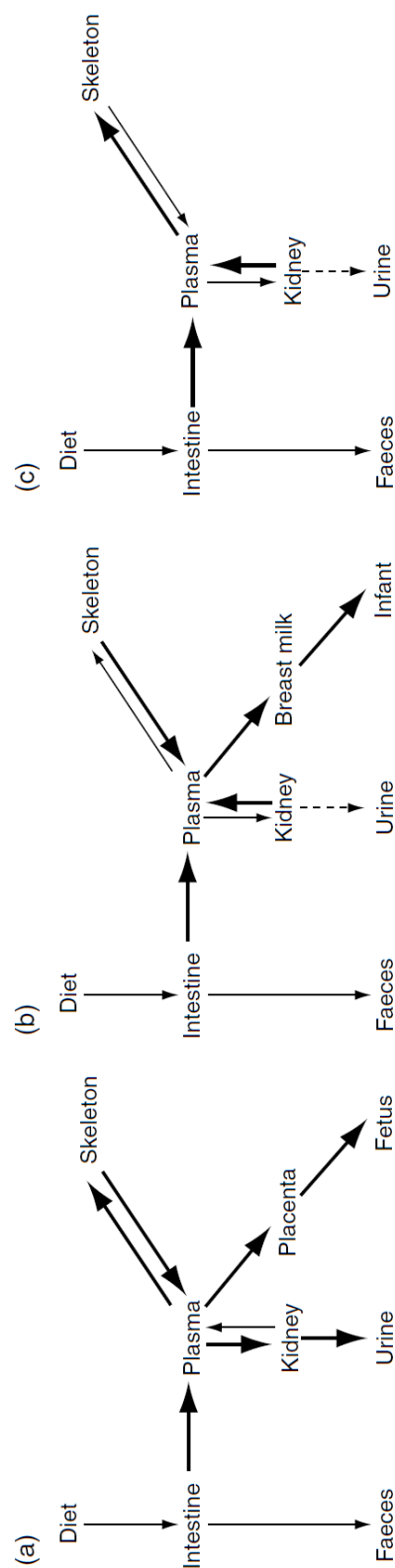
Fig. 1: Schematic diagrams summarizing differences in calcium flux, compared with non-pregnant, non-lactating women (NPNL), during pregnancy (a), lactation (b), and post lactation (c). Thicker arrows denote an increase from NPNL; dashed arrows denote a decrease from NPNL (Olausson et al., 2012, p.44).

Chapter 3

General methods

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Animals

Skeletal samples from female and male baboons used in this thesis are sourced from the Southwest National Primate Research Center (SNPRC). The majority of baboons housed at the SNPRC are olive baboons (*Papio hamadryas anubis*), with additional colonies of hamadryas baboons (*Papio hamadryas hamadryas*), red baboons (*Papio hamadryas papio*), yellow baboons (*Papio hamadryas cynocephalus*), as well as hybrids. The animals analyzed in the present study are all olive baboons (*Papio hamadryas anubis*), yellow baboons (*Papio hamadryas cynocephalus*), and their hybrids. During life all animals were housed outdoors in social group housing (**Fig. 1**) and were fed a commercial monkey chow to which they had ad libitum access. Animal care personnel and staff veterinarians at Texas Biomedical Research Institute (TBRI)/Southwest National Primate Research Center (SNPRC) provided daily maintenance and health care to all animals. All procedures related to their treatment during their lives at the TBRI were approved by the Institutional Animal Care and Use Committee in accordance with established guidelines. All animals used in this study were sacrificed for reasons unrelated to this project (e.g. culling) or died naturally during the period of 2004-2008. Complete clinical records for each animal were checked to be certain that animals with significant medical conditions known to alter normal bone metabolism (e.g. diabetes, chronic renal disease, ovarian cancer) were excluded from study.

Reproductive Histories

Several components of a female baboon's reproductive history are recorded at the SNPRC and are relevant to this thesis. Only sexually mature adult females are used in this study. Menarche typically occurs between age 3 and 4 years at the SNPRC (Glassman et al., 1984; McGill et al., 1996) with first parturition occurring approximately 1 year later for captive *Papio* (Birrell et al., 1996). Progeny born to SNPRC females are listed in a database for each baboon, and from this list **Parity** (defined as **total number of offspring produced per dam**) is calculated for this study. All full-term offspring were included in this calculation, including stillbirths and neonates that died soon after birth.

Lactation times are not directly measured at SNPRC but are available indirectly by accessing detailed mother-infant room location data. Mothers and infants are kept together in the same room location during the breastfeeding period (**Fig. 2**) and are separated after weaning. For the purpose of this study, total lactation time per female is interpreted as the total number of days mother-infant pairs are located together in the same room. Weaning has occurred when offspring are moved to a new location independently of the mother. *Papio h. spp.* at the SNPRC typically breastfeed their young 6-12 months, with older mothers sometimes tolerating a more extended period of juvenile dependency (Dr. Linda Brendt 2014, personal communication). In

this thesis, the measurement of lactation stress chosen was percent lifetime lactation (**%LifeLact**) and was calculated for each female by **adding the total number of days spent breastfeeding all offspring, divided by the dam's age (in days) x 100.**

A related variable, **interbirth interval (IBIavg)**, or **the average number of days between successive pregnancies,** is a significant component of a female's lifetime reproductive success. In wild baboon populations, this measurement is variable and is significantly altered by environmental conditions and the physiological state of the mother. A typical IBI for wild *Papio h*. is 24 months (Sigg et al., 1982), a period which includes gestation, nursing, and the several months required for the mother to regain body fat stores and resume cycling. At the SNPRC where females have ad-libitum access to food and water, IBIavg is more related to the viability of the infant. In captive colonies, IBI for *Papio h*. is approximately 13 months based on normal offspring growth and survivability (Birrell et al., 1996). However, if an infant is stillborn or dies soon after birth, the interbirth interval is shorter, approximately 11 months in both wild (Altmann et al., 1977) and captive (Gauthier, 1999) populations. Postpartum amenorrhea, the period of time between the birth of an infant and the resumption of cycling, is also short (29-39 days) following stillborn offspring in both wild and captive *Papio* populations (Altmann et al., 1977; Smuts and Nicolson, 1989; Gauthier, 1999; Cary et al., 2002). However in normally breastfeeding captive *Papio*, postpartum amenorrhea is longer—approximately 5.5 months (Gauthier, 1999). For the present study, **IBIavg** was calculated by **adding the total number of days between successive pregnancies and dividing this by the total number of offspring born to a dam**.

In general, female fecundity at the SNPRC tends to increase with age as females mature, and subsequently decline later in life. In contrast to wild populations where fecundity is affected by seasonably variable food and environmental conditions, *Papio* fecundity at the SNPRC is relatively unaffected by external factors. Fecundity is normally high until age 18-19 years when most females begin to experience irregular cycles (Martin et al., 2003), and both the frequency of irregular cycles and the average interbirth intervals gradually increase after age 20 years with complete cessation of cycling occurring by age 26 years (Martin et al., 2003). In general, births become rare after age 20 years in captivity and have not been recorded at all in baboons over age 24 years (Honoré and Carey, 1998).

Bone structure and remodeling

The basic division in bone tissue is between cortical and trabecular bone. Cortical and trabecular bone differ from each other structurally, biomechanically, and seem to play different roles in mineral metabolism. Cortical (or compact) bone is dense and forms the hard outer shell of all bones and is particularly thick in some regions, i.e in the shafts of long bones. Compact bone appears to the naked eye as a solid continuous mass of skeletal tissue—inherent spaces and structures of cortical bone can only be visualized with a microscope (Steiniche and Hauge, 2003). Trabecular (or cancellous) bone has an open spongy structure and is found in marrow-rich regions of the skeleton, i.e. inside the distal regions of long bones and inside the bodies of vertebrae. This bone is less dense than cortical bone and is composed of thin trabeculae, or bony spicules, organized in a 3-D lattice structure. Trabecular bone is not homogenous and the number, thickness, and size of trabeculae differ depending on the bone, and even within an

individual bone—this feature is often attributed to bone's biomechanical function (Steiniche and Hauge, 2003).

Lamellar bone is the type of organization that predominates in both cortical and trabecular bone after childhood bone growth is completed (Einhorn, 1996). Lamellar bone is arranged into organized parallel sheets or bundles of lamellae. The main structural unit of lamellar bone is the osteon. Groups of osteons form Haversian systems. Early in growth a first set of osteons develops and are called "primary" osteons. Subsequent osteons, formed throughout the rest of life, are referred to as secondary osteons. Primary osteons differ from secondary in that their central canal contains two or more blood vessels and these osteons do not have a delimiting cement line or any interstitial lamellae surrounding it (Hall, 2005). A secondary osteon contains a larger central canal with only a single blood vessel (Hall, 2005) and is oriented in the long axis of tubular bone (Steiniche and Hauge, 2003). In adult human cortical bone, around two thirds of the volume is composed of secondary osteons (Steiniche and Hauge, 2003) with the remainder being constituted by interstitial bone, the remnants of pieces of previous secondary osteons, as well as a few layers at the two major surfaces, termed subperiosteal and subendosteal circumferential lamellae (Steiniche and Hauge, 2003). The central canals, or Haversian canals, flow together and are linked with the periosteum and with the marrow cavity though transverse and oblique canals known as Volkmann's canals. Within the osteon unit are mature bone cells, osteons, that communicate with each other via canaliculi, small cytoplasmic projections; canaliculi also provide the route for cell nutrition and link these cortical osteocytes with those on the very outside layer of bone (the periosteum). Secondary cancellous bone also contains osteons but they are organized as broad bands of parallel lamellae, with some interstitial lamellae as remnants of old secondary osteons (Steiniche and Hauge, 2003).

In addition to the osteocyte, there are two other major bone cells involved in the repair and formation of the osteon—the osteoblast and osteoclast. Osteoblasts are cells that line the bone-forming surface, derived from other cells in close proximity called osteoprogenitor cells (Walsh et al., 2003). The primary function of the osteoblast is to lay down a new layer of bone; they do so by synthesizing membrane associated alkaline phosphatase and by regulating the deposition of bone matrix molecules (i.e. type I collagen and non-collagenous proteins) (Walsh et al., 2003). Osteoblasts eventually mature into osteocytes (surrounding a Haversian canal) after they've been encased in a mineral matrix called a lacuna (Neuman and Neuman, 1980). Osteoclasts are the other major cell type. Osteoclasts are derived from the monocyte/macrophage family (Chambers, 2000; Lerner, 2000) and are large and multi- nucleated. Osteoclasts perform the task of resorbing and the mineralized matrix that surrounds it (Walsh et al., 2003). During this process, the osteoclast forms a "ruffed membrane" and secretes photons and hydrolases that can degrade both the organic and inorganic components of bone (Walsh et al., 2003).

Osteoblasts, osteoclasts and osteocytes work in concert through an anatomic structure called the Basic Multicellular Unit (BMU) (Frost, 1969). The components of the BMU function to remove old bone (via osteoclasts) and subsequently refill the excavated surfaces (via osteoblasts), leaving behind final mature bone cells (osteoclasts) organized into an osteon unit. A BMU can travel and carve through the long axis of bone over a span of several months, always keeping its constituents organized in the same spatial and temporal relationship to a central capillary (Parfitt, 2003). As the BMU moves through anatomical space it forms a roughly

cylindrical resorption space. Following resorption, lamellar bone is deposited in concentric layers of lamellar bone eventually refilling up the resorbed area until only a central vascular canal remains; this central vessel in its infancy will provide progenitor cells for the advancing BMU and later will act as a route for mineral exchange between bone and the bloodstream and will finally supply nutrients to the fixed osteocytes around the periphery of the final osteon (Parfitt, 1994a; Enlow and Hans, 1996; summarized in McFarlin et al., 2008). The ultimate result of the BMU's work is a secondary osteon, separated from the surrounding bone tissue by a reversal cement line (McFarlin et al., 2008). The number of active BMUs and the relative amounts of bone resorbed and formed within individual BMUs determine the rate of bone turnover (Dempster and Zhou, 2006; Eriksen, 1986).

Histomorphometry Overview

Bone histomorphometry is the histological examination a decalcified bone sample performed in order to assess quantitative information on bone modeling, remodeling, and microstructure. There are two primary modes through which to assess histomorphometry: 1) dynamic and 2) static. Dynamic histomorphometry involves the injection of labeling agents (e.g. tetracycline) into the live subject prior to bone biopsy acquisition. These labeling agents highlight areas of new bone formation (e.g. recent osteoblastic activity) on a bone surface, which allows for these regions to be visualized and quantitatively analyzed (Moreira-Kulak and Dempster, 2010). Static histomorphometry assesses bone remodeling and microstructure without the visualization of effector cells (BMU), though accurate estimations of BMU activity can still be assessed through algorithmic composite bone measures (e.g. activation frequency and bone formation rate) (Frost, 1987a). The analysis in this dissertation utilizes static histomorphometry of thin bone sections. Basic histomorphometric variables are derived from primary measurements made at the microscope, such as area (e.g. Haversian area), perimeter, and thickness (e.g. cortical thickness). Composite measures (e.g. osteon population density) are more sophisticated assessments of secondary osteonal remodeling, and are calculated using means from basic histomorphometric variables.

The femur and humerus midshafts were chosen as the sites of cortical histomorphometric analysis for this study. Prior research has shown that substantial bending strains are generated at the midshaft of appendicular sites (e.g. humerus and femur) during locomotion (Biewener 1991). As a result, the midshaft long bone diaphysis is a common site of bone structural and histomorphometric analysis in both human and non-human primate studies of bone remodeling (Burr, 1992; Goldman et al., 2003; Havill et al., 2013; Paine and Godfrey, 1997; Schaffler and Burr, 1984; Schaffler et al., 1985; McFarlin et al., 2008; Thomas et al., 2005). The midshaft point is ideal for several reasons (Burr, 1992: 180), primarily because this site:

1) can be accurately defined in different animals.
2) is mechanically relevant and "comparable" across species.
3) can be mechanically analyzed using beam theory even prior to collection of relevant in vivo strain data.

In this study, standard methods for characterizing cortical bone microstructure in the absence of dynamic in vivo bone labeling were used to measure histomorphometric variables related to intracortical remodeling (Abbott et al., 1996; Stout, 1978). Although most studies do not analyze secondary osteon subtypes (i.e. Type I, II, drifter, etc) separately, the large prevalence in drifter osteons observed in this sample population was interesting and worthy of further analysis. Drifting osteons are characterized by "a Haversian system in which there is continuous resorption on one side and continuous formation on the other. As a result the system becomes flattened in one plane and transversely to this plane, and in effect 'waltzes' through the cortex" (Frost, 1964; p 151). Structurally mature drifting osteons (**Fig 3a**) exhibit a non-centrally located Haversian canal surrounded by 4+ concentric lamellae on one side, creating a hemicyclic lamellar "tail" (Robling and Stout, 1999). This is in contrast to stationary, "non-drifting" osteons which deposit lamellae evenly on all sides of a Haversian canal (**Fig 3b**). 3-D analyses of drifting osteons have revealed that these osteons have a morphologically distinct BMU model than those of non-drifting osteons (Robling and Stout, 1999). Presently it is unclear why these osteons are created and what function they have in relation to remodeling. Drifting osteons have been found in the cortices of individuals from all ages (Epker and Frost, 1965) and seems to be unrelated to bone ontogenic processes. Mechanical strain distributions also fail to explain why drifting BMUs change drift directions so frequently and extensively throughout their history (Lacroix, 1971; Burton et al., 1989; Robling and Stout, 1999). An additional hypothesis suggests that the primary stimulus for drifting osteons is related to mineral homeostasis (Coutelier, 1976). However, drifting osteons often drift back onto their undermineralized "tails" and it is unclear why this subtype would preferentially resorb undermineralized bone if the primary function was related to mineral homeostasis (Epker and Frost, 1965). Osteoclasts tend to avoid undermineralized bone and osteoid (Jowsey and Gordan, 1971), so it is unlikely that drifter BMUs are activated in times of additional mineral needs.

The hypothesis that drifting osteons may be activated during times of increased calcium needs is further tested in this thesis, using measurements of drifter osteons in combination with known reproductive histories. Because drifting osteon parameters are unstandardized in the literature, the typical terminology for area osteon measurements (On.Ar, H.Ar) was adapted for this specific morphological subtype (Dh.Ar, DOn.Ar). Whenever possible, measurement names and abbreviations of such adhere to the system of nomenclature, standards and units described in Parfitt et al., (1987). The derived porosity and percent cortical area measurements used here are taken from Agnew and Stout, (2012). In this study, the difference between %C/T and %C_A/T was calculated to evaluate the effects of porous spaces on bone area measurements. The traditional measurement of cortical area (Ct.Ar) cannot account for the intracortical bone loss that occurs with high porosity and bone area may be erroneously reported if measurements incorporating porosity are not reported (Agnew and Stout, 2012). All basic and composite measures and their calculations are detailed below:

1. **Osteon area** (μm^2, On.Ar): defined as the total area circumscribed by the reversal (cement) line, averaged across all osteons within the field of assessment.

2. **Haversian** (central) **canal area** (μm^2, H.Ar): defined as the total area of the Haversian canal, averaged across all canals within the field of assessment.

3. **Osteon population density** (#/mm^2, OPD), (osteon number + osteon fragment number)/bone area): defined as the number of secondary osteons with intact Haversian canals plus the number of osteon fragments (i.e. the number of secondary osteons in the field of view that are without intact Haversian canals) normalized by the total bone tissue assessed.

4. **Drifter osteon area** (μm^2, DOn.Ar): defined as the total area circumscribed by the most recent/proximal reversal (cement) line for each drifter osteon, averaged across all drifter subtype osteons within the field of assessment.

5. **Drifter osteon Haversian canal area** (μm^2, Dh.Ar): defined as the total area of the drifter osteon Haversian canal, averaged across all drifter osteon canals within the field of assessment**.**

6. **Percent osteonal bone** (%On.B), (osteonal area/bone area) x 100): defined as the proportion of the observed cortex occupied by secondary osteons with intact Haversian canals, expressed as a percentage.

7. **Percent porosity area** (%Po.Ar) (total void areas including Volksman's canals, Haversian canals, immature osteons with unfilled portions, and other pores/ bone area) x100): defined as the ratio of intracortical void areas (including central, longitudinal canals and other porous spaces but excluding osteocyte lacunae) to total assessed bone area, expressed as a percentage.

8. **Percent cortical area** (%C/T) (Ct.Ar/Tt.Ar x 100): defined as cortical area relative to the total area, expressed as a percentage.

9. **Percent absolute cortical area** (%C$_A$/T) (Ct.Ar$_A$/Tt.Ar x 100; Ct.Ar$_A$= Ct.Ar-Po.Ar in mm^2): defined as absolute cortical area (minus porosity) relative to the total area, expressed as a percentage.

In addition to histomorphometrics, midshaft cross-section area measurements were also taken. With aging, bone models and resorbs regionally along the periosteum and endosteum of the diaphysis, which results in alterations to cortical and medullary areas. Large changes to relative cortical and medullary areas can have profound effects on area of moment inertia, geometric properties, and subsequent bending strength (Bouxsein, 2001). Although human studies have shown some trends in midshaft geometry changes by age and sex (Thomas et al., 2005; Bousson et al., 2000; Riggs et al., 2004), there is also a high level of individual variation in the rate and extent of cortical bone loss and apposition (Feik et al., 1997; Bell et al., 1999a; Stein et al., 1999). Cross-sectional quantity and the distribution of diaphyseal cortical bone is plastic throughout the life cycle, especially in response to the changing biomechanical loads to which it is subjected (e.g. Lanyon, 1982; Cowin, 1989; Trinkaus et al., 1994). Details of the cross-sectional area measurements and their calculations, as used in the present study, are summarized below:

1. **Total area** (mm^2, Tt.Ar): defined as the total area of the bone midshaft, including cortical and endosteal components. The outer margin (periosteal border of the cortice) is traced and the Bioquant software determines the total area for the midshaft specimen.

2. **Cortical area** (mm^2, Ct.Ar) (Tt.Ar-Es.Ar): defined as the cross-sectional area of cortical bone between the periosteal and endosteal surfaces. Cortical area is manually calculated by subtracting the total area from the endosteal area.

3. **Endosteal area** (mm^2, Es.Ar): defined as the area of the marrow cavity. The inner margin (endosteal border) is traced and the Bioquant software determines the endosteal area for the midshaft specimen.

Midshaft sample preparation

During the period of 2004-2008 (at the SNPRC), *Papio* humeri and femora were collected at the time of animal necropsy, wrapped in saline-soaked gauze, placed in air-tight plastic bags and were kept frozen until (recent) sample preparation. The midshaft point (total length divided by 2) was calculated for both humerus and femur samples. In a small number of males (n=7), the deltoid ridge of the humerus was especially robust and the inferior portion extended to the midshaft point. Because the muscle attachment could interfere with cross-sectional area and intracortical remodeling variables, a section of bone was removed 5cm below the stopping point of the deltoid ridge. This diaphyseal location was similar to the midshaft section taken from the other males and females included in analysis.

Epiphyseal fusion was complete in all individuals. Humeri samples were taken from a total of 109 individual animals (females = 87; males=22), and femora samples were taken from a total of 76 individual animals (females = 49; males =27). The femur histological slides were already prepared by Dr. Havill and were borrowed for use in this study. The humerus samples were prepared during the course of this dissertation, using a similar methodology as defined in Havill et al., (2013). Details are further outlined below.

Slide Preparation and analysis

A 10mm midshaft block was cut from each humerus bone using a band saw. From this block, two slices of ~500 μm were removed using an Isomet saw and placed into labeled cassettes. These cassettes were temporarily placed into a container of 70% EtOH until the time of further processing. Each ~500 μm section was manually ground down (with sandpaper) until the desired thickness of ~100 μm was reached. The final ~100 μm bone slice was fixed to a labeled glass slide using epoxy glue. After 24 hours of drying, a coverslip was glued (with epoxy) onto the bone surface for each slide.

Thin-sections were analyzed for histomorphometry using a combination of polarized and plane light, using a hilfsobject red 1st order quartz compensator (Olympus model U-P521) with a Leica DM 2500 upright microscope and QImaging Micropublisher 5.0 RTV digital camera. Direct measures of intracortical remodeling dynamics were obtained using Bioquant Osteo v 7.20 (R&M Biometrics, Nashville, TN) in real time. All measurements were recovered via manual selection and/or tracing. The cortex was sampled (**Fig. 4**) by reading along eight 1mm rays (1, anterior; 2, antero-medial; 3, medial; 4, medo-posterior; 5, posterior; 6, postero-lateral; 7, lateral; 8, antero-lateral). The selected ray surfaces equates to approximately 50% of the total cortex surface area. Analysis was conducted at 100x magnification.

Macroscopic cross-sectional area measurements of the midshaft humerus and femur slides were done under 8x magnification with the assistance of Bioquant image analysis software. Prior to measurement, Bioquant was calibrated to 8x magnification using a standardized glass micrometer so that a pixel to length ratio could be established. Thin section slides were placed under a Lecia MZ6 dissecting scope with a QImaging Micropublisher 5.0 RTV digital camera attached. Manual tracings of the periosteum and endosteum were used to calculate cross-sectional area variables.

Trabecular bone architecture analysis (Micro-CT)

Trabecular bone is a continuous 3-D network of bars and plates, its microstructure composed primarily of vertical columns of bone connected by horizontal struts (Mosekilde, 1993; Parfitt, 1992). Trabecular bone is highly heterogeneous; in addition to varying densities it possesses varying orientations and degrees of orientation. The vertebral body site has traditionally been preferred for sampling as a proxy for whole-body cancellous bone health, in particular lumbar sites rather than thoracic or caudal spinal segments, because the volume of the lumbar vertebral bodies is the greatest and offers the most micro-architectural network to sample (Bouxsein et al., 2010). Trabecular microstructural variability (e.g. bone quality) has a significant effect on mechanical properties and tissue stress in humans (Yeni et al., 2008) and is important to measure alongside bone volume (e.g. bone quantity). Human vertebral cancellous bone is one of the primary sites for age-related bone loss. Trabecular bone microstructure, density, and biomechanical properties all change with age, and typical alterations include reduced cortical thickness and density, decreased plate number, lower fractional volume of plate-life bone, and decreased trabecular connectivity (Walker et al., 2013). Other aspects of human biology, including population and ancestry (Yeni et al., 2011, Rui et al., 2013) are also major sources of microstructural variation. As demonstrated in Chapter Two, lumbar trabecular bone volume is significantly decreased during the periods of pregnancy and lactation (e.g. Olausson et al., 2008; More et al., 2001; Laskey and Prentice, 1999), though the long-term effects of this restructuring on subsequent microstructure is not yet well understood. The micro-CT assessment of lumbar trabecular architecture in the present study will help clarify if reproductive history is also a significant source of variation in shaping microstructure.

Although there are many methods through which to assess trabecular bone health (e.g. histomorphometry, DXA, pqCT), micro-CT (μCT) is ideal because it offers high-resolution

assessments that reflect both quantitative and qualitative aspects of trabecular bone. Bouxsein et al., (2010) summarizes the advantages of using μCT to assess trabecular bone as the following:

(1) It allows for direct 3D measurement of trabecular morphology, such as trabecular thickness and separation, rather than inferring these values based on 2D stereologic models (Hildebrand and Luegsegger 1997a, 1997b; Laib et al. 1997), as is done with standard histologic evaluations
(2) Compared with 2D histology, a significantly larger volume of interest is analyzed
(3) Measurements can be performed with a much faster throughput than typical histologic analyses of histomorphometric parameters using undecalcified bone specimens
(4) Assessment of bone morphology by μCT scanning is nondestructive; thus samples can be used subsequently for other assays, such as histology or mechanical testing.

The primary hypothesis is that reproductive histories will be informative in explaining bone volume and trabecular micro-architectural variation throughout the baboon lifespan. This study uses standardized measures of trabecular architecture, including bone volume, trabecular thickness, trabecular separation, trabecular number, connective density and the structural model index (Parfitt et al., 1987). These measures reflect both quantitative and qualitative aspects of trabecular bone.

1. **BV/TV**, bone volume fraction **(%)**: defined as cancellous bone volume, or the percent of total marrow cavity that is occupied by cancellous bone (both mineralized and non-mineralized). Low= deficit in bone mass

2. **Tb.Th**, trabecular thickness **(μm)**: defined as the mean distance across individual trabeculae

3. **Tb.N**, trabecular number **(1/mm):** defined as the average number of trabeculae per unit length

4. **Tb.Sp,** trabecular separation **(μm):** defined as the mean distance between trabeculae

5. **Conn.D**, connectivity density **(1/mm^3):** defined as the degree (or redundancy) of trabecular connectivity

6. **SMI**, structure model index: defined as the characterization of the structure of trabeculae as either rod-like or plate-like. SMI will be 0 for parallel plates and 3 for cylindrical rods (Hildebrand and Ruegsegger, 1997)

L1 sample acquisition and processing

During the period of 2004-2008 (at the SNPRC) frozen vertebral spines (the segment of T12-L7) were taken at animal necropsy, wrapped in saline-soaked gauze, placed in air-tight plastic bags and were kept frozen until sample preparation. During the summer of 2012, I traveled to the SNPRC and located the frozen vertebral columns for each baboon chosen in the dissertation sample population. A small percentage of the animals did not have vertebrae taken at necropsy or had severely damaged (crushed) lumbar vertebrae. From the remaining samples, the L1 vertebra was selected for inclusion in this thesis. Soft-tissues (muscles, tendons and ligaments) surrounding the spinal column were first dissected away so identification of L1 was possible. This isolated vertebra and was cut away from the rest of the vertebral column using a band saw.   Selected vertebrae were immediately wrapped in saline-soaked gauze, placed into labeled air-tight plastic bags, and frozen. At the end of the summer, the vertebrae were placed into a Styrofoam box filled with dry ice and overnight FED-EX mailed to the U.C. Berkeley Anthropology Department.

The cylindrical core sampling methodology was selected for the assessment of trabecular bone micro-architecture. Cylindrical cores are preferred because they are a precise and standardized means of bone sampling and cores can subsequently be used in mechanical strength testing for further analysis (e.g. Yeni et al., 2011; Yeni et al., 2008; Hansen et al., 2009; Hou et al., 1998). Lumbar height and length was measured for all female L1 samples and a core size of 8mm x 12mm was selected as an appropriately scaled region of interest. Diamond-coated coring drill bits were ordered from Starlite Tools (800-727-1022).   All coring work was performed in the Orthopaedic Biomechanics Laboratory (PI: Dr. Tony Keaveny) at U.C. Berkeley. Frozen L1 samples were oriented superiorly (i.e. with the articulation for T12 facing upward) with lamina facing anteriorly and midpoint was calculated using the total length across the superior surface of the vertebra (from left to right) as well as the total width (anterior to posterior). From this midpoint, the center of the core was taken 5mm laterally to the right in order to avoid the vertebral artery that supplies blood to the interior of the vertebral body. Coring was performed continuously under water to reduce friction.

Micro-CT core preparation and analysis

A total of n=24 vertebral L1 cores from females with variable ages, parities and %LifeLact were analyzed at the J.D. Wheat Veterinary Orthopedic Research Laboratory, School of Veterinary Medicine on the U.C. Davis campus. Due to the high sample costs and lengthy reconstruction times of micro-CT analysis, a female-only pilot sample of n=24 was deemed appropriate for the budgetary and time constraints of this doctoral thesis.

A cylinder of approximately 8 mm in diameter and 12 mm in lengthwas centered in a scaffold core and was imaged (70 kVp, 114 µA, 300 ms integration time, average of 3 images) using a high- resolution µCT specimen scanner (µCT 35, Scanco Medical; Bassersdorf, Switzerland). 820 contiguous slices of 2,048 x 2,048 pixels were imaged with 10 µm resolution and slice thickness (voxels). "Voxel" refers to the discrete unit of the scan volume that is the result of the tomographic reconstruction. Differences in voxel size (eg, 10 to 20 mm) have little

effect on the evaluation of structures with relatively high thickness (ie, 100 to 200 mm), such as trabeculae in humans or large animal models (Bouxsein et al., 2010). On each of the 2-dimensional images a region of interest was manually drawn. The region of interest excluded the external rim of the core to avoid inclusion of broken and partial trabeculae pieces resulting from the coring process. Serial tomograms were reconstructed from raw data of 1000 projections per 180 degrees using a cone beam filtered back projection algorithm from Feldkamp (et al., 1984). The tomograms were calibrated to 0.0, 99.6, 200.0, 401.0 and 800.3 mgHA/cc concentrations of hydroxyapatite (HA) so that grey-values of the images were converted to units of density in mgHA/cc.

Bone tissue in the reconstructed images (**Fig. 5)** was determined by thresholding (191-3000 mgHA/cc) to partition mineralized tissue from fluid and soft-tissues. After thresholding, the image noise was reduced using a low-pass Gaussian filter (σ=.8, support=1). Bone volume fraction (**BV/TV**) was determined by dividing the number of pixels representing bone tissue (BV: bone volume) by the number of pixels in the cylindrical segment (TV: total volume). The mean density of all material in the volume is apparent bone mineral density (aBMD). The mean density of only the mineralized material is the tissue mineral density (TMD). Trabecular number **(Tb.N)**, mean trabecular thickness **(Tb.Th)**, and mean trabecular separation **(Tb.Sp)** were calculated using a direct morphometric analysis (Hildebrand et al., 1999; Odgaard and Gundersen, 1993; Hildebrand and Ruegsegger, 1997). Connectivity density **(Conn.D)** was calculated by dividing the connectivity measure by TV, where connectivity is the maximum number of trabeculae that can be broken before the specimen is separated into two parts (Odgaard and Gundersen, 1993). Values from all continuous slices were averaged for each core and this average was used in all subsequent calculations and analyses.

Statistics

All tests were performed using IBM SPSS Statistics v21. Differences were considered significant if p<0.05.

1.	Overall descriptive statistics, including sample mean, range, standard (SD), and standard error of the mean (SEM) were assessed for each parameter in this study. Scatterplots were created in order to assess statistical outliers and observe overall trends prior to further statistical testing. Data normality was examined using the Shapiro-Wilk test in addition to sample skewness and kurtosis assessments. Homogeneity of variance within age, sex, and reproductive variable groups was tested to compare the relative variabilities of these subsets.

2.	In **Chapter Four**, data normality tests (the Shapiro-Wilk test as well as skewness and kurtosis) within both sexes were first assessed to determine the appropriateness of parametric analyses. Afterward, further descriptive statistics and t-tests were carried out to explore the significant differences in cortical remodeling between males and females at both the humerus and femur sites. Stepwise multiple linear regression analyses were performed to investigate the relationships between histomorphometric bone variables, age, sex, and body

weight. Microstructural and cross-sectional area parameters were entered as dependent variables in regression models and age, body weight, and sex were used as independent variables. Extent of variance explained by the model was assessed by R^2, with strength of the model was explained by a significant p-value of <.05.

3. In **Chapter Five**, data normality tests (the Shapiro-Wilk test as well as skewness and kurtosis) were first assessed to determine the appropriateness of parametric analyses. Scatterplots helped identify statistical outliers and overall trends. Exploratory statistics were carried out to assess sample ranges and means for reproductive variables in two age cohorts: young adults (aged 6-18 years) and old adults (aged 19-33 years). The mean age of onset of irregular monthly cycling and peri-menopausal phenomena is reported to be 19.0 years in SNPRC baboons (Martin et al., 2003), therefore females were grouped into two separate reproductive cohorts for analysis: a normally cycling, young adult group, and an irregularly cycling, older adult group (similar to a pre-menopausal cohort). Descriptive statistics and t-tests were performed to show differences in remodeling variables and cross-sectional area measurements by site (midshaft humerus and femur) in females only. Stepwise multiple linear regression analyses were performed to investigate the relationships between cortical bone variables, age, body weight, and reproductive history variables. Microstructural and cross-sectional area parameters were entered as dependent variables in regression models and age, body weight, parity, IBIavg, and %LifeLact were used as the independent variables. For each analysis, all females (per site sample) were analyzed as a whole group first to observe the overall effect across all ages, and then assessed separately as young and old age cohorts to account for the relative effects of age and hormonal differences. One-way ANCOVAs (with age held constant) were examined in the more robust humerus groups but not the femur groups due to small sample sizes (i.e. <10 per group in an ANCOVA). Tukey's test (a post hoc test that accounts for multiple comparisons) was used to determine which pairs of groups differed significantly when the p-value for a parameter was less than 0.05. The purpose of these ANCOVAs was to elucidate if high/low/0 parity, and high/low/0 %LifeLact groups of females differed significantly in any of the cortical bone parameters.

4. In **Chapter Six**, data normality tests (the Shapiro-Wilk test as well as skewness and kurtosis) were first assessed to determine the appropriateness of parametric analyses. Several variables did not pass normality tests (BV/TV, SMI, Tb.Th) and nonparametric tests were used subsequently to analyze them. Scatterplots helped identify statistical outliers and general trends in trabecular architecture parameters with age, sex, and reproductive variables. Descriptive statistics and t-tests (or Mann-Whitney tests for non-normal variables) were performed to show significant differences in trabecular variables between young and old age groups. Linear regressions were carried out to assess if reproductive history was

significant in explaining variation in trabecular architecture. Cross-method bivariate correlations (using Pearson's tests for normal variables and Spearman's tests for non-normal variables) were carried out to assess the relatedness of cortical and trabecular microstructural patterns.

Fig. 1: Baboon social housing at the SNPRC

Fig. 2: Baboon mother and infant pair at the SNPRC
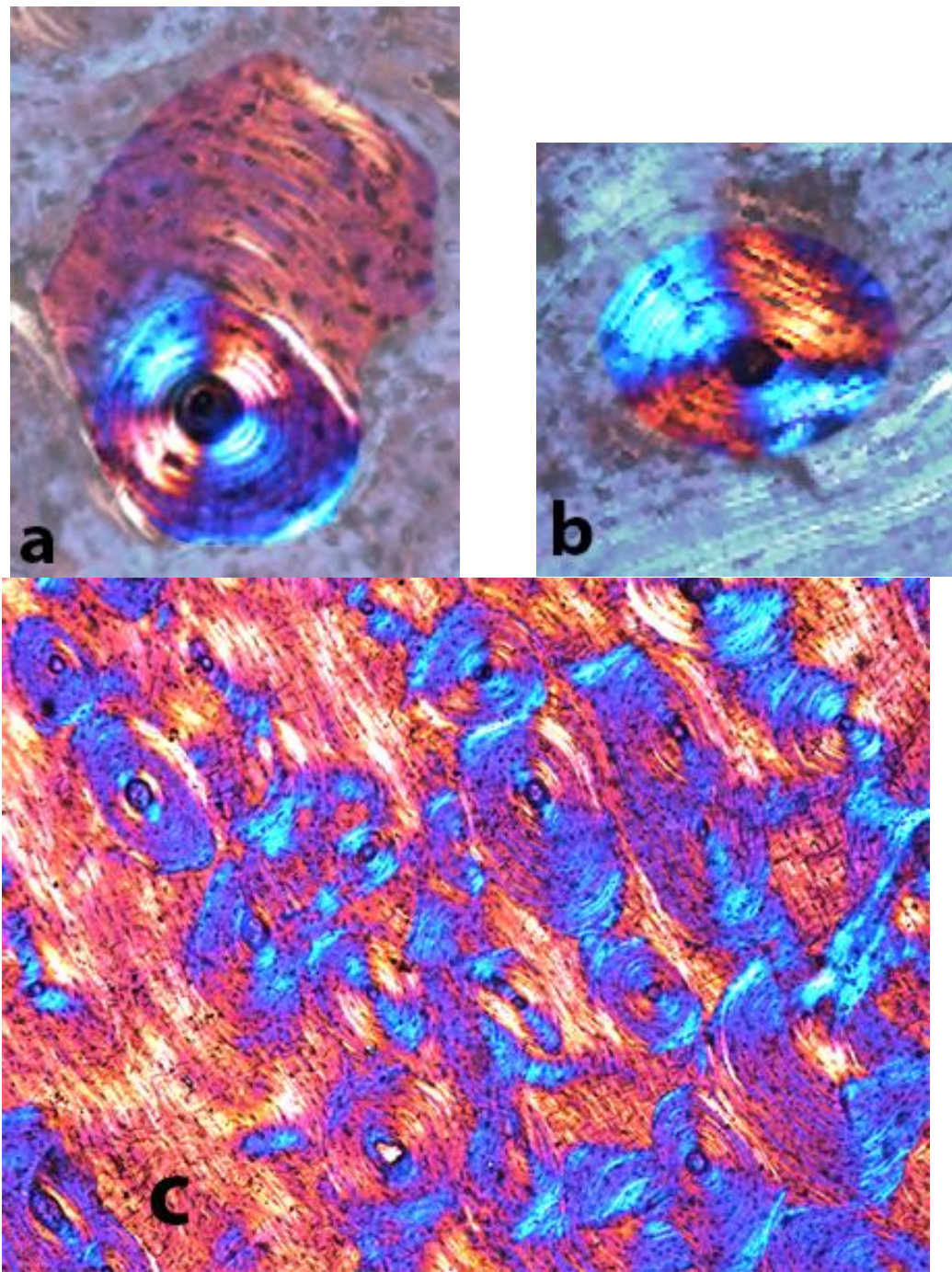
Fig. 3: Cortical remodeling of the baboon appendicular midshaft. **a)** example of a drifting osteon. The dark blue aspect represents the current proximal location of the osteon. The light orange aspect represents the "tail," showing the past drifting history across the cortex. **b)** example of a stationary (non-drifting) secondary osteon. **c)** example of typical remodeling seen in a histological section
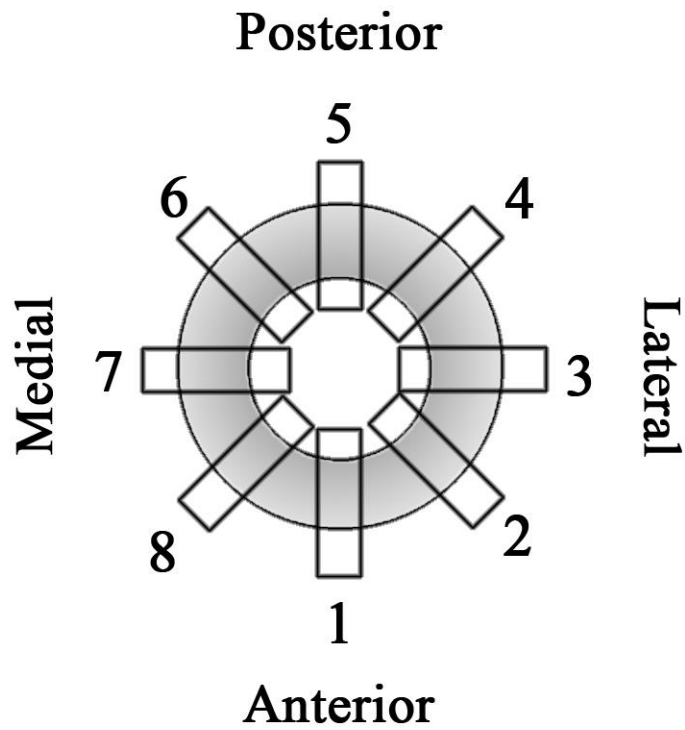
Fig. 4: Histomorphometric sampling methodology for the midshaft humerus and femur (adapted from Havill, 2003; Havill et al., 2013); rays denote sampled area of the cortex.
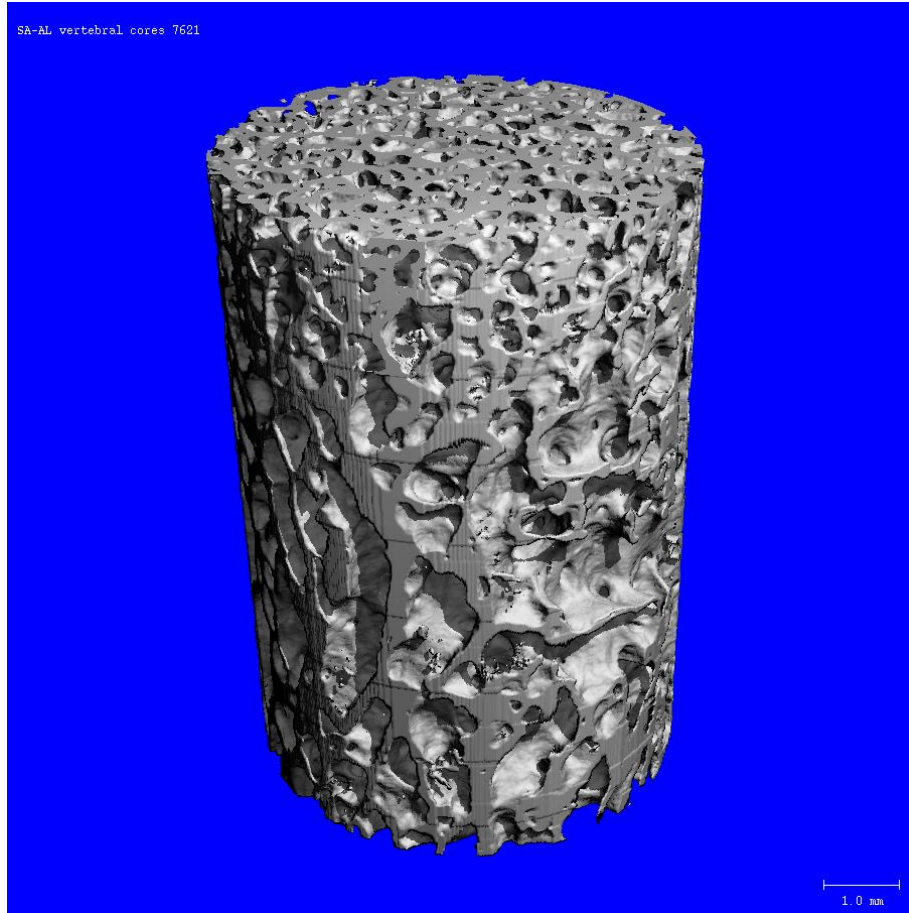
Fig. 5: 3-D reconstruction of the micro-CT analysis of L1 core sample

Fig 6: Summary of cortical, trabecular, and reproductive history variables

Histomorphometry

Abbrev.	Name	Definition
On.Ar	Osteon area	Mean osteon area
H.Ar	Haversian area	Mean Haversian area
DOn.Ar	Drifter osteon area	Mean drifter osteon area
Dh.Ar	Drifter Haversian area	Mean drifter Haversian area
%On.B	Percent osteonal bone	Percentage of the cortex occupied by osteonal bone
OPD	Osteon population density	Number of intact secondary osteons + osteon fragments/ bone area
%Po.Ar	% Porosity	Percentage of the cortex occupied by intracortical void areas
%C/T	Percent cortical area	Percent cortical area relative to total area
%C_A/T	Percent absolute cortical area	Percent cortical area (- porosity) relative to total area

Cross-Sectional Area

Abbrev	Name	Definition
Ct.Ar	Cortical area	Cortical area (from periosteum to endosteal border)
Es.Ar	Endosteal area	Area of the medullary space
Tt.Ar	Total area	Total area of the midshaft

Trabecular Architecture

Abbrev	Name	Definition
%BV/TV	Bone volume fraction	Percent cancellous bone volume/total volume
Tb.Th	Trabecular thickness	Mean trabecular thickness
Tb.N	Trabecular number	Mean trabecular number
Tb.Sp	Trabecular separation	Mean trabecular separation
Conn.D	Connectivity density	Degree of trabecular connectivity
SMI	Structure Model Index	Characterization as either rod or plate-like trabeculae

Reproductive History

Abbrev	Name	Definition
Parity	Number of live offspring	Total offspring number born to a dam
%LifeLact	Percentage of life spent lactating	Total percentage of dam's life spent lactating
IBIavg	Average interbirth interval	Average time between live offspring for a dam

Chapter 4

Age, sex, and body weight-related intracortical secondary remodeling dynamics in the midshaft humerus and femur of baboons (*Papio hamadryas*).
_____

Introduction

Bone is a heterogenous, dynamic tissue that ultimately reflects intricate relationships between a myriad of factors, including phylogeny, ontogeny, genetics, mechanics, and life-history. Skeletal tissues are plastic, continually being remodeled and renewed over the lifetime of an organism. Remodeling of mammalian bone is highly variable within and between skeletal sites and serves various different functions. Analyses of secondary intracortical remodeling within and among mammalian taxa can also provide insight into bone's relationship to various aspects of life history, including mechanical loading, aging, genetics, and hormonal changes. The majority of studies of intracortical remodeling in macaques and baboons have thus far focused on age-related dynamics (Burr, 1992; Havill, 2003a; Havill et al., 2013) or on the extent to which patterns of secondary intracortical remodeling in adult animals varies in relation to species-specific locomotor patterns (Paine, 1994; Paine and Godfrey, 1997; Schaffler and Burr, 1984, McFarlin et al., 2008). Some recent work in captive macaques and baboons has focused on the extent to which genes explain variation in some intracortical remodeling parameters (Havill, 2003a; Havill et al., 2013).

Osteoblasts, osteoclasts and osteocytes work in concert through a temporary anatomic structure called the Basic Multicellular Unit (BMU) (Frost, 1969). As the BMU moves through anatomical space it forms a roughly cylindrical resorption space. Following resorption, lamellar bone is deposited in concentric layers of lamellar bone eventually refilling up the resorbed area until only a central vascular canal remains; this central vessel in its infancy will provide progenitor cells for the advancing BMU and later will act as a route for mineral exchange between bone and the bloodstream and will finally supply nutrients to the fixed osteocytes around the periphery of the final osteon (Parfitt, 1994a; Enlow and Hans, 1996). The ultimate result of the BMU's work is a secondary osteon, separated from the surrounding tissue by a reversal cement line. Proximal factors influencing BMU activation are not entirely understood, but research has shown several influences, including hormones, circulating levels of calcium and other minerals, and mechanical stimuli (e.g., Martin et al., 1998; Riggs et al., 2002; works in Seibel et al., 1999). Bone remodeling results in a change in the number of osteons per unit area and in the replacement of older bone with new, secondary osteonal bone (Burr and Martin, 1989)

Aging

Tissue age is a major determinant of bone fatigue and accumulated micro-damage. Degradation of skeletal tissues and generation of micro-damage becomes more pronounced with increasing numbers of loading cycles experienced, as bone approaches the end of its fatigue life (i.e., number of cycles to failure) (Martin et al., 1998) and is further accelerated by high strain

loading (Burr et al.,1985; Carter and Caler, 1985; Carter et al., 1981; Schaffler et al., 1989). Because osteon remodeling is cumulative over the lifetime, it is typical for variables like osteon population density (OPD), osteon number (On.n), osteon fragment number (Fg.n), and percent osteonal bone (%On.B) to increase with age once bone growth and formation has been completed (Stout, 1992; Stout and Lueck, 1995). Osteon population density, osteon number, and percent osteonal bone are all ways of quantifying degree of remodeling in cortical bone. Osteon population density measures the total number of osteon creations (intact secondary osteons as well as fragments) averaged over the total area assessed and conveys information about overall remodeling activity. A similar measure, osteon number, simply calculates the total number of intact osteon creations observed within a bone section, but does not provide information about how this number relates to the total cortical area—to assess this, you would need percent osteonal bone, which calculates the percentage of cortical bone (analyzed) that is composed of secondary osteons.

Within old world monkeys, age related adult intracortical remodeling at appendicular sites has been the most studied in macaques (Burr, 1992; Havill, 2003a), and baboons (Havill et al., 2013). These species exhibited some age-related variability in several parameters related to this study, including osteon number, population density, and osteon size. In a study of femoral intracortical remodeling in macaques (n=54), Burr, (1992) found overall weak effects of age in osteon size (r^2=.15), OPD (r^2=.07), and %On.B (r^2=.07). Havill, (2002) also examined femoral intracortical remodeling in macaques (n=75) and found a stronger effect of age on OPD (r^2=.23) (Havill, 2002) in skeletally mature individuals. It is unclear why OPD showed a stronger effect in Havill, (2002), but is likely related to the larger sample of skeletally mature adults. In a study of femoral intracortical remodeling in baboons (n=101, *Papio hamadryas spp*), age accounted for 18% of variance in (OPD), 21% of variance in wall thickness (W.Th) and 9% of porosity (%Po.Ar) (Havill et al., 2013).

Non-existent or weak age-effects have also been observed in percent area of secondary osteonal bone (%On.B) in old world monkeys. In a study utilizing both juveniles and adults, "age class" category did significantly account for variation in percent secondary osteonal bone (%HAV, a measure similar to %On.B) in the midshaft femur (61%) and humerus (47%) in C. aethiops (McFarlin et al., 2008), suggesting that age may have a stronger effect on %Hav during the growth process and juvenile period. However, the only study utilizing genotyped animals (*Papio h.*) observed a strong effect of genetics in determining midshaft femur %On.B (75% variation explained), with age only accounting for 9% of total variance (Havill et al., 2013). If %On.B is under significant genetic control in all old world monkey species, this would explain the observed weak effect of adult age in explaining %On.B variance (Burr, 1992; Havill et al., 2013).

Sex

Previous studies of intracortical remodeling in catarrhine primates have typically found little difference between males and females. No significant sex-effect was seen in remodeling dynamics in the midshaft and proximal femur sites of *Macaca mulatta* (Burr, 1992; Havill, 2002). Sex also did not account for significant variation seen in the proportion of osteonal bone (% On.B.) observed at the midshaft femur and humerus of *C. aethiops* and *H. lar* (McFarlin et

al., 2008). However, this is unsurprising considering both *m. mulatta* and *C. aethiops* are not sexually dimorphic in body size and weight (Burr, 1992). In the more sexually dimorphic *Papio hamadryas,* sex*age was a significant factor in explaining H.Ar (13%) and W.Th (21%) in the midshaft femur. Large sex differences in body weight will influence bone strain and mechanical loading at the midshaft, and will likely influence midshaft cortical remodeling. Body weight is included as a covariate in this present study and is tested for significance in influencing remodeling at the midshaft.

Body size

Because the majority of previous work has focused on comparing non-human primate remodeling dynamics by taxa, there is little data available on how within-species differences in body weight variance affects remodeling variation. Two studies comparing remodeling rates among multiple species of non-human primates revealed a tendency for larger-bodied taxa to intracortically-remodel their femora and humeri to a greater extent than smaller-bodied taxa (Paine, 1994; Paine and Godfrey, 1997), however differences in positional behavior have also been significant in accounting for interspecific variation (Paine, 1994; Paine and Godfrey, 1997; Schaffler and Burr, 1984), complicating the body size-remodeling relationship.

Humerus and femur site differences

There is some evidence to support the theory that appendicular forelimb and hindlimb remodeling is related to species-specific locomotor behavior and local substrate biomechanics. In a comparative sample of primates (*Galago senegalensis, Otolemur crassicaudatus, Macaca fascicularis, Macaca mulatta, Macaca arctoides, Erythrocebus patas,* and *Cercopithecus aethiops*) found that humeral and femoral midshaft have independent patterns of remodeling (Paine, 1994; Paine and Godfrey, 1997) and that %On.B in the femur and humerus varied by general categories of locomotor behavior. Relative femoral to humeral remodeling was lowest in quadrupeds, compared to leapers and slow climbers who exhibited high rates of femoral remodeling. The more terrestrial species of quadrupeds examined, vervet monkeys (*C. aethiops*) and patas monkeys (*Erythrocebus patas*), exhibited the highest index of humeral to femoral intracortical remodeling of the species examined (Paine, 1994; Paine and Godfrey, 1997). However, in another comparative study, both *C. aethiops* (a partially terrestrial quadruped) and *H.lar* (an arboreal brachiator), both exhibited a significantly greater midshaft humeral to femoral %HAV (McFarlin et al., 2008). The mechanics of both terrestrial quadrupedalism and fast arboreal brachiation may both stimulate increased remodeling of the midshaft humerus, but this issue is not resolved.

Study Goals

The present study seeks to characterize appendicular intracortical remodeling dynamics in a captive baboon species (*Papio hamadryas*) by basic life history parameters (age, sex, and body weight) at two midshaft sites (humerus and femur). Although the effects of age, sex, and

genetics have already been quantified at the femoral midshaft in another study of baboon intracortical remodeling dynamics (Havill et al., 2013), this study focuses on the site differences in intracortical remodeling and cross-sectional area seen between the humerus and femur. The literature indicates that there are significant differences in taxa-specific remodeling patterns between the midshaft humerus and femur in primates (Paine, 1994; Paine and Godfrey, 1997, McFarlin et al., 2008), though the reasons why are still unclear. Within-species site differences have also not been well characterized, since most studies in primates focus on differences between taxa. The primary hypotheses of the study are as follows: **1)** baboon intracortical remodeling patterns will show site-specific patterns and will differ significantly between the midshaft humerus and femur, and **2)** the sexual dimorphism in baboons will be associated with significant intra-site differences in remodeling and cross-sectional area; as a result, sex and body weight effects will significantly explain much of the difference at both the humerus and femur.

Methods

Right humeri and right femora were obtained from baboons at necropsy and kept frozen until sample analysis. Humeri and femora from 87 females and 22 males (humeri), and 49 females and 27 males (femora) were assessed in this study. The midshaft point of each long bone was calculated for both humeri and femora samples and was used as the site for investigating intracortical remodeling in this study. The midshaft location is commonly chosen for investigations of bone structural properties and histocompositional variables, making results more comparable to other published non-human primate studies (e.g., Burr, 1992; Goldman et al., 2003; Havill et al., 2013; Schaffler and Burr, 1984; Schaffler et al., 1985, McFarlin et al., 2008). Slide preparation methodology is detailed in Chapter 3.

Although most studies do not analyze secondary osteon subtypes (i.e. Type I, II, drifter, etc) separately, the larger prevalence in drifter osteons (see **Fig. 1**) observed in this sample population was interesting and worthy of further analysis by age, sex, and site. Standard methods for characterizing cortical bone microstructure in the absence of in vivo fluorocrome labeling were used to measure the following histomorphometric variables related to intracortical remodeling (Abbott et al., 1996; Stout, 1978). The porosity and percent cortical area formulae used here are taken from Agnew and Stout (2012). In this study, the difference between %C/T and %C_A/T was calculated to evaluate the effects of porous spaces on bone area measurements. The traditional measurement of cortical area (Ct.Ar) cannot account for the intracortical bone loss that occurs with high porosity and bone area may be erroneously reported if measurements incorporating porosity are not reported (Agnew and Stout, 2012). Whenever possible, measurement names and abbreviations of such adhere to the system of nomenclature, standards and units described in Parfitt et al., (1987). However, drifting osteon measurements are unstandardized in the literature; as such, the previous terminology for area measurements (On.Ar, H.Ar) was adapted to reflect differences in these two morphological subtypes:

1. **Osteon area** (μm^2, On.Ar): The total area circumscribed by the reversal (cement) line averaged across all osteons within the field of assessment.

2. **Haversian** (central) **canal area** (μm^2, H.Ar): The total area of the Haversian canal averaged across all canals within the field of assessment.

3. **Osteon population density** (#/mm^2, OPD), (osteon number + osteon fragment number)/bone area: The number of secondary osteons with intact Haversian canals plus the number of osteon fragments (the number of secondary osteons in the field of view that are without intact Haversian canals) normalized by the total bone tissue assessed.

4. **Drifter osteon area** (μm^2, DOn.Ar): The total area circumscribed by the most recent/proximal reversal (cement) line for each drifter osteon, averaged across all drifter subtype osteons within the field of assessment.

5. **Drifter osteon Haversian canal area** (μm^2, Dh.Ar): The total area of the drifter osteon Haversian canal averaged across all drifter osteon canals within the field of assessment**.**

6. **Percent osteonal bone** (%On.B), (osteonal area/bone area) x 100: The proportion of the observed cortex occupied by secondary osteons with intact Haversian canals

7. **Percent porosity area** (%Po.Ar) (total void areas including Volksman's canals, Haversian canals, immature osteons with unfilled portions, and other pores/ bone area) x100: The ratio of intracortical void areas (including central, longitudinal canals and other porous spaces but excluding osteocyte lacunae) to total assessed bone area.

8. **Percent cortical area** (%C/T) (Ct.Ar/Tt.Ar x 100): cortical area relative to the total area, expressed as a percentage.

9. **Percent absolute cortical area** (%C_A/T) (Ct.Ar$_A$/Tt.Ar x 100; Ct.Ar$_A$= Ct.Ar-Po.Ar in mm^2): absolute cortical area (taking into account porosity) relative to the total area, expressed as a percentage.

Macroscopic measurements of the midshaft humerus and femur include:

1. **Total area** (mm^2, Tt.Ar): the total area of the bone midshaft, including cortical and endosteal components: The outer margin (periosteal border of the cortex) is traced and the Bioquant software determines the total area for the midshaft specimen.

2. **Cortical area** (mm^2, Ct.Ar) (Tt.Ar-Es.Ar): cross-sectional area of cortical bone between periosteal and endosteal surfaces. Cortical area is manually calculated by subtracting the total area from the endosteal area.

3.   **Endosteal area** (mm^2, Es.Ar): area of the marrow cavity. The inner margin (endosteal border) is traced and the Bioquant software determines the endosteal area for the midshaft specimen).

Statistical analysis

Data normality tests (the Shapiro-Wilk test as well as skewness and kurtosis) were first assessed to determine the appropriateness of parametric analyses. Exploratory statistics were then carried out to assess ranges and means for ages, remodeling parameters and cross-sectional area measurements by midshaft site (humerus and femur) and are summarized in **Tables 1**, **2**. These data combine both females and males together to elucidate site-specific patterns; p-values indicate significant differences between the sites (<.05). Descriptive statistics and t-tests exploring differences between the sexes at both sites are summarized in **Tables 3-6**.

Stepwise multiple linear regression analyses were performed to investigate the relationships between microstructural bone variables, age, sex, and body weight (**Tables 7-12**). Microstructural and cross-sectional area parameters were entered as dependent variables in regression models and age, body weight, and sex were used as independent variables. All statistical analyses were performed using SPSS 21.0 (SPSS).

Results

Overall sample characteristics:

Both site samples share an age range of approximately 4-33 years of age, with an average age of 17 years and are not statistically different (p=.989, **Table 1**). Males and females differ significantly in body weight (p=.000), with males weighing on average 28kg and females 17kg (**Table 3-4**).

Site differences in intracortical remodeling:

T-tests (**Table 1**) indicate several significant differences in intracortical remodeling between the humerus and femur. Osteon area (On.Ar), Haverisan area (H.Ar), and %C$_A$/T are all significantly larger in the femur. Interestingly, drifting osteon parameters (DOn.Ar, Dh.Ar) are both larger in the humerus. This indicates that there is significant dimorphism in osteon subtype dynamics between the two sites, with larger non-drifting osteons dominating the femur and larger drifting osteons in the humerus. Porosity (%Po.Ar) and percent osteonal bone (%On.B) are both larger in the humerus (**Fig. 2**), with the higher proportion of remodeled bone contributing to the larger amount of porosity (**Table 13**). Haversian and Volkmann's canals (which supply blood to osteons) have previously been observed as the major contributors to intracortical porosity (Martin and Burr, 1984). %C$_A$/T (percent cortical area adjusted for porosity) is significantly lower in the humerus and is correlated with the high proportion of porosity seen at this site (**Table 13**). Porosity has a negative relationship with %C$_A$/T (the more

porous a bone is, the lower the total %C_A/T). Together, the high %Po.Ar, high %On.B and low %C_A/T characterize the midshaft humerus as significantly more remodeled than the femur. Significantly more remodeled midshaft humerus over femur cortices have previously been observed in several terrestrial primate taxa (Paine, 1994; Paine and Godfrey, 1997; McFarlin et al., 2008). In this study, age, sex, and body weight account for much of the variation seen within the sites. These results are further summarized below.

Sex effects on intracortical remodeling

Within the two sites, significant sex differences in remodeling are seen. In the humerus, On.Ar (μ= 8340.27, *p* .000), H.Ar (μ= 671.63, *p* .000), and DOn.Ar (μ=11275.32, *p* .000) are all larger in males (**Table 3**). Interestingly, though osteon and Haversian sizes are significantly smaller in females, OPD (μ= 9.95, *p* .000) and %On.B (μ= 7.94, *p* .042) are both significantly larger (**Table 3**). This suggests that there is significant sexual dimorphism in osteon dynamics in the humerus. Males grow larger osteons but have fewer of them and the reverse is true for females. Because OPD (**Fig. 3**), %On.B (**Fig. 2**), and %Po.Ar (**Fig. 4**) are all significantly higher in females, this results in a significantly lower total %C_A/T (μ= 49.64, *p* .000)(**Fig. 5**) (correlations shown in **Table 14**). Unsurprisingly, stepwise regressions found sex to be the most significant dependent variable in the humerus in determining OPD (r2= .17, p=.000), On.Ar (r^2=.28, p=.000), DOn.Ar ($r^{2=}$ .20, p=.000), H.Ar (r^2=.13, p=.000)(**Table 7**), as well as %Po.Ar (r^2= .40, p=.000) and %C_A/T (r^2=31, p=.000) (**Table 8**). This makes sense in light of the significant sex differences seen between males and females in these intracortical remodeling variables.

Significant sex effects were also seen in the femur. In the midshaft femur, H.Ar (μ= 677.30, p=.000)(**Fig. 6**) and Dh.Ar (μ= 667.19, p=.026) were both significantly larger in females (**Table 4**). Significantly larger H.Ar in baboon females (p=.001) was previously reported in Havill et al. (2013). Though sex differences were seen in osteon areas in the humerus, this was not seen in the femur. Sex was also not a significant factor in determining osteon size in humans at the anterior third of the femur (Britz et al. 2009). However, porosity does show a sex effect at the midshaft femur: %Po.Ar (μ= 22.80, p=.007) is higher in females and as a result, %C_A/T (μ= 53.79, p=.021) is lower (**Table 4**). High porosity and low %C_A/T in females is a trend observed at both midshaft sites (**Tables 3-4**). Unsurprisingly, stepwise regression models found significant sex effects in H.Ar (r^2=.21, p=.000) (**Table 10**) and %Po.Ar (r^2=.097, p=.006) (**Table 11**). Significantly larger Haversian sizes in females at the midshaft femur are significantly correlated with %Po.Ar (**Table 17**).

Age effects on intracortical remodeling

Age weakly predicts variation in intracortical remodeling at the midshaft humerus and femur, and this is a pattern confirmed by other studies of femoral intracortical remodeling in macaques and baboons (Burr 1992; Havill 2002; Havill et al. 2013). In the both the humerus and femur, age has a negative relationship with %C/T (*humerus* r^2=.064, p=.008, Table 8; *femur* r^2=.122, p=.002, **Table 11**). Approximately 35% of humerus %C_A/T variance (p=.000)(**Table 8**)

was explained by a regression model in which sex and age were combined, though sex alone explained 31% of %C_A/T variance (p=.000) so the overall contribution of age is small. Age also shows a weakly negative relationship with %C_A/T (r^2= .086, p=.010)(**Table 11**) in the femur. Also in the femur, age shows a weak effect on On.Ar (r^2=.083, p=.011) (**Table 10**). Modest age effects on femoral osteon size have been observed in macaques (r^2=.15; Burr 1992), baboons (r^2=.20, *age*sex*; Havill et al., 2013) as well as in humans (r^2=.28; Britz et al., 2009).

Body weight *effects on intracortical remodeling*

Overall, body weight is not a significant predictor of intracortical remodeling at either midshaft site. In the femur, it only weakly explains the variance observed in Dh.Ar (r^2= .064, p=.027) (**Table 10**), and in the humerus it contributes slightly to OPD (approximately 5.9%, when the effects of sex are subtracted) (**Table 7**). Haversian canal size is a result of cessation in refilling, and smaller Haversian canals may indicate that cortical bone was less metabolically active at the time of death (Pfeiffer et al., 2006). Body weight was also observed to have some effect on the individual vigor of osteoblasts and osteoclasts through osteon size in macaques (Burr, 1992). However, the effect here (6%) is weak and the majority of Haversian canal size is clearly related to other unexamined variables. Body weight is also associated with increased OPD, and may have a slightly stimulatory effect on BMU activation, but again, this effect is small (6%)(**Table 7**). Small effects of body weight on OPD were also observed in macaques (r^2=.10; Burr 1992)

Cross-sectional area:

Midshaft humerus and femur cross-sectional areas differ significantly, with femur areas significantly larger in all parameters examined: Tt.Ar (μ= 13.37, p=.001), Ct.Ar (μ= 9.30, p=.001), Es.Ar (μ= 4.07, p=.001) (**Table 2**). Significant sex effects were also observed at both sites: at both the humerus and femur, females showed significantly smaller areas in all parameters (**Tables 3, 6**). Multiple regression analyses helped to clarify further how cross-sectional area is affected by sex differences, age, and weight.

In both the humerus and femur, total variation in Tt.Ar is nearly completely explained by a combined regression model of sex, age, and weight together (*humerus* r^2=.801, p=.000, **Table 9**; *femur (*r^2=.740, p=.000, **Table 12**), though the effects are not equally shared; sex alone accounts for the vast majority of variance in midshaft Tt.Ar (*humerus* r^2=.753, p=.000, **Table 9**; *femur* r^2=.652, p=.000 **Table 12**). In both the humerus and femur, a significant amount of variation is explained by a combined model of sex and weight (*humerus* r^2=.818, p=.000, **Table 9**; *femur* r^2=.757, p=.000, **Table 12**), though again sex alone accounts for the majority of variation explained (*humerus* .783, p=.000 **Table 9**; *femur* r^2=.706, p=.000, **Table 12**) and the added effects of weight are small. In both the humerus and femur, a combined model of sex and age accounts for the most variation in Es.Ar (*humerus* r^2=.442, p=.000, **Table 9**; *femur* r^2=.379, p=.000, **Table 12**), again with sex alone accounting for most of the variance (*humerus* r^2=.380, p=000, **Table 9**; *femur* r^2=.249, p=.000, **Table 12**). Overall, sex explains the most variation seen in cross-sectional area at both sites, with age contributing some influence in Tt.Ar and Es.Ar, and

body weight contributing some influence in Tt.Ar and Ct.Ar.   The significant contribution of sex (and not primarily body weight) in determining cross-sectional area is related to the differential influences of hormones and sex-specific growth trajectories during puberty (see discussion).

Discussion

Results of the present study support the hypotheses that intracortical remodeling differs significantly by site and sex. Site-specific patterns include significantly larger drifter osteon measurements (DOn.Ar, Dh.Ar) in the humerus and larger stationary osteon measurements (On.Ar, H.Ar) in the femur (**Table 1**). The differences in osteon sizes may be related to the kinesiology of hindlimb vs. forelimb mechanics during quadrupedal walking. Previous work has shown that pronograde primates tend to place approximately 60% of their weight on the hindlimbs (Kumura et al., 1979), and this percentage increases during periods of fast gait (Reynolds, 1985a,b). In quadrupedal primate species, the forelimbs participate less in propulsion and instead have increased function in braking and steering (Schaffler et al., 1985). There is some evidence to suggest that increased loading can result in larger osteon size through targeted microcrack repair. Larger osteons are more susceptible to microfractures and tend to undergo targeted remodeling (Burr, 2002; Martin, 2000, 2002, 2003), which could positively skew the average osteon size for the midshaft femur. However, osteon size is also scaled allometrically with cortical area (Paine, 1994), therefore it is possible that the larger On.Ar (**Table 1**) in the midshaft femur is also simply a scaling effect of the significantly larger Ct.Ar at that site (**Table 2**). In the humerus, the larger sizes of drifting osteons is also interesting and worth further investigation. Previous studies have suggested that drifting osteon movement is related to strain exposure (Frost, 1964; Martin et al., 1974; Lanyon and Bourn, 1979; Hert et al., 1994) but it is unclear how strain gradients affect the size of these osteons. Further experimental work is needed to clarify the relationship of appendicular midshaft bending strains and drifting osteon size. %Po.Ar is significantly higher in the midshaft humerus (**Table 1**), and is correlated with a significantly lower %C_A/T (%C_A/T is negatively correlated with %Po.Ar) (**Table 13**). %Po.Ar in the humerus is increased in part by large %On.B (**Fig. 2**), OPD, and increased Dh.Ar size (**Table 14**). The overall number and areas of Haversian canals has been previously observed as the major contributors to intracortical porosity (Martin and Burr, 1984). Together, the high %Po.Ar, high %On.B and low %C_A/T (**Table 1**) characterize the midshaft humerus as significantly more remodeled than the femur. The increased humeral to femoral %On.B in this study is unsurprising, and is an observation that has been reported in terrestrial quadrupedal primate species by several authors (Paine, 1994; Paine and Godfrey, 1997; McFarlin et al., 2008).

In this study, much of the difference in within-site remodeling is explained by sex. At the midshaft humerus, males showed significantly larger On.Ar, H.Ar, DOn.Ar, and lower %On.B and OPD (**Table 3**). This results in an interesting humerus-specific sexual dimorphism in osteon dynamics: males grow larger osteons and Haversian canals but have lower overall activation of remodeling whereas females have increased secondary osteon activity (OPD, %On.B)(**Fig.s 2-3**) but smaller osteons and Haversian areas (**Table 3**). OPD rates and secondary osteon sizes are linked in several ways. In general, the relative diameter of the osteon will indicate the timing of the transition from osteoclastic activity (resorption) to osteoblastic activity (deposition) (Pfeiffer et al., 2006). As a result, smaller osteons could reflect a higher BMU activity, as the creation of

new secondary osteons will take less time if they are smaller. There are several reasons why this could occur: 1) a heightened activation rate could reflect a need to strengthen fatigue-damaged bone, or 2) temporarily reduce bone mineralization (Pfeiffer et al., 2006). It is possible that female baboons have an increased need to strengthen fatigue-damaged humeral bone as a result of differences in physical activity rates and infant-rearing behaviors. Infant holding, carrying, and nursing behaviors could result in increased female humeral loading, likely from the added weight associated with picking up and carrying young toward the front of the body, as well as the corresponding size increase in forearm musculature and periosteal apposition. Another possibility is that the increased fetal and neonatal calcium demands during multiple cycles of pregnancy and lactation cause reduced bone mineralization in the cortex, decreasing osteon and Haversian area sizes and increasing OPD. The significant effect of sex in explaining %Po.Ar and %C_A/T in both the midshaft humerus and femur is likely related to the unique calcium demands of reproducing females, and overall increase in cortical resorption and remodeling. Increased %Po.Ar has strongly negative effects on bone fracture toughness (Yeni et al., 1997), compressive fracture (Ebacher et al., 2007) and bone strength (McCalden et al., 1993) in the femur and other appendicular sites. Further testing is needed to clarify what factors are responsible for increasing porosity in baboon females. The importance of female reproductive history in driving intracortical remodeling dynamics is explored in Chapter 5 of this thesis.

Overall at the midshaft humerus, sex explains 17% of variance in OPD, 28% of variance in On.Ar, 20% of variance in DOn.Ar, and 13% of H.Ar (**Table 7**). However, sex explains much less variance in the femur, where the only sexually dimorphic trait in osteon dynamics observed was H.Ar (**Table 10**), which is larger in females both here (**Fig. 6**) and in another study of baboon remodeling (Havill et al., 2013). It is possible that the significant mechanics of propulsion in the femur lead to much less overall remodeling variation between the sexes. Genetics may also play a role; at the midshaft femur, On.Ar and %On.B show strong heritability in baboons (Havill et al., 2013).   Overlap in sex effects between the midshaft femur and humerus is seen in %Po.Ar and %C_A/T, where in both, females show significantly higher %Po.Ar and significantly lower %C_A/T (**Tables 3, 4**). Increased female %Po.Ar in the midshaft humerus (**Fig. 4**) is significantly correlated with increased OPD (**Table 14**), whereas increased %Po.Ar in the femur is significantly correlated with female H.Ar (**Table 17**). It is also entirely possible that other large, non-haverisan resorption spaces are significant in increasing female femoral %Po.Ar. Measurements of large pores (typically near the endosteum) are included in this study's calculation of porosity, but are not assessed statistically in isolation (as Dh.Ar and H.Ar are). In aging human women, midshaft femur trabecularization of the endocortical region (in the form of porous resorption spaces) plays a large role in determining %Po.Ar (Zebaze et al., 2010), and approximately 50% of cortical bone loss at peripheral sites is the result of pores within the cortex adjacent to the marrow. Failure to account for the trabecularization at the endosteal border results in a three-fold underestimation of porosity (Zebaze et al., 2010). One of the strengths of the present study is that it does account for endosteal pores, likely giving a more encompassed view of %Po.Ar changes between the sexes.

Here, sex also exhibits strong effects on cross-sectional area measurements at both the midshaft femur and humerus. Sex alone accounts for 75% (*humerus*) and 65% (*femur*) of variation seen in Tt.Ar (**Tables 9, 12**), 78% (*humerus*) and 71% (*femur*) of variance in Ct.Ar (**Tables 9, 12**), and 38% (*humerus*) and 25% (*femur*) in Es.Ar (**Tables 9, 12**). Significant effects

of sex were previously reported in a study of cross-sectional geometry in baboons (Hansen et al., 2009), where sex accounted for ~56% of variance in cross-sectional area (CSA), minimum (I_{MIN}) and maximum (I_{MAX}) principal moments of inertia, and polar moment of inertia (J). These significant sex differences are unsurprising in light of differential hormonal influences during growth and maturation. Larger femoral midshaft means in baboon males is observed here and in Hansen et al., (2009). Males tend to develop wider bones in the appendicular skeleton (even after adjustment for height) because of high testosterone driving larger periosteal apposition during puberty (Vanderschueren et al., 2004). During adulthood in men, cortical thickness and cross-sectional area both continue to increase, and this trend is not seen in women (Ohisson et al., 2011; Walsh et al., 2012). Together, these sex-specific patterns could explain the significantly larger Tt.Ar and Ct.Ar means seen in baboon males over females at both midshaft sites. Previous studies have also shown that with aging in both men and women, endocortical resorption typically outpaces periosteal growth (resulting in an overall reduction in the cortice), though this process occurs much faster in women than in men (Burghardt et al., 2010; Lauretani et al., 2008; Duan et al., 2003; Riggs et al., 2004). The age and sex-specific patterns of endocortical resorption (**Fig. 7**) could explain why a regression of age and sex together is associated with increasing Es.Ar here in baboons. Age and sex together account for approximately 44% of Es.Ar variation in the humerus (**Table 9**) and 38% in the femur (**Table 12**). The age-related thinning of the cortex in proportion to Tt.Ar also explains the observed negative relationship of age with %C/T seen at both sites (**Tables 8, 11**). However at both sites, body weight shows a slight positive relationship with cortical area (**Tables 9, 12**), and the associated periosteal apposition may help to slow the effects of age-related cortical thinning (Ruff and Hayes, 1988).

Conclusion

This study observed several significant differences in intracortical remodeling with respect to midshaft site and biological sex. Drifting osteon measurements are significantly larger in the humerus (DOn.Ar, Dh.Ar), whereas stationary osteons are larger in the femur (On.Ar, H.Ar). The reasoning for this unknown but may relate to the difference in forelimb vs. hindlimb mechanical loading in the baboon. The humerus is also significantly more porous, showing high %Po.Ar, high %On.B, and low %C$_A$/T. This characterizes the midshaft humerus as significantly more remodeled than the femur, an observation that has been reported in terrestrial quadrupedal primate species by several authors (Paine, 1994; Paine and Godfrey, 1997; McFarlin et al. 2008). Here, intracortical remodeling shows stronger sex effects in the humerus than in the femur. However, at both sites, females exhibit significantly higher %Po.Ar and lower %C$_A$/T, and this may relate to the skeletal resorbtion typically seen during pregnancy and lactation. Female cortical bone quality and quantity with respect to reproductive history is further explored in the following chapter.
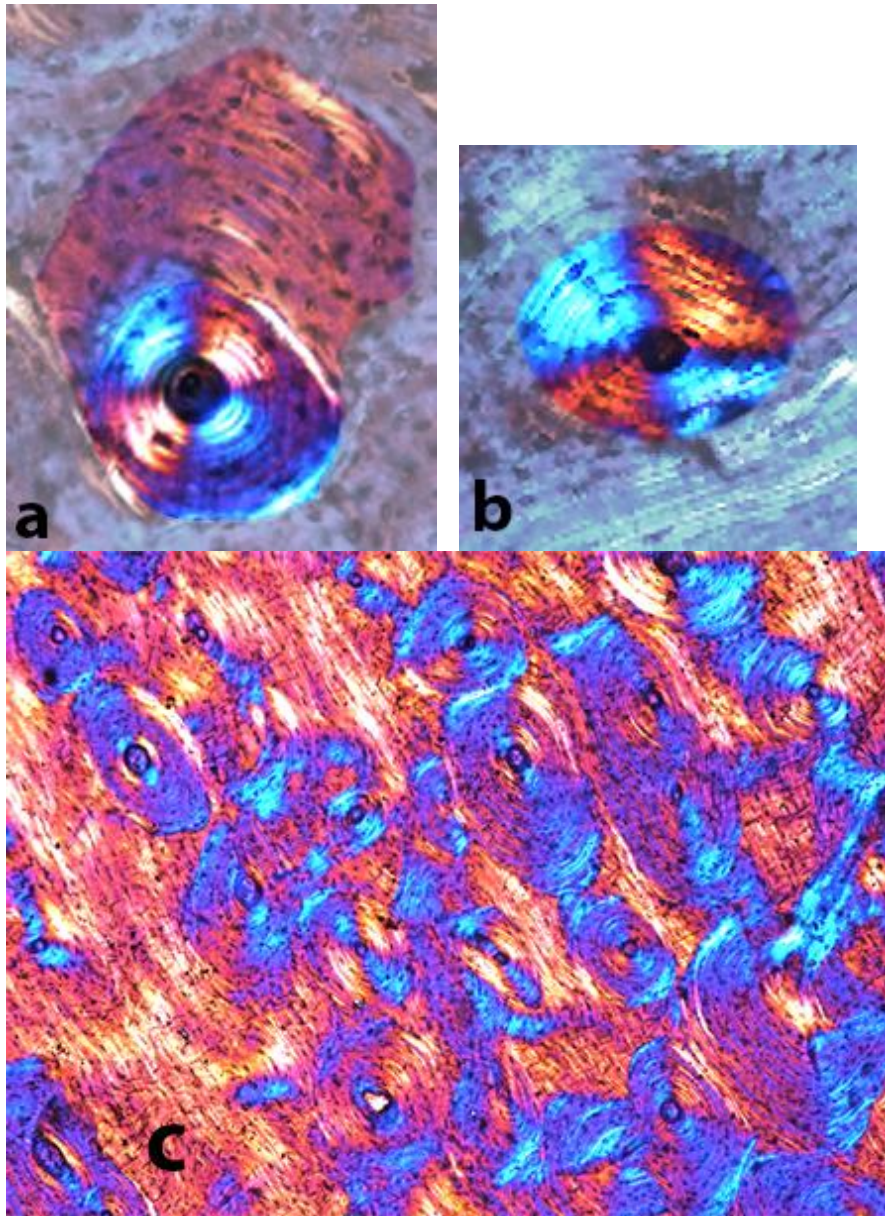
Fig. 1: Cortical remodeling of the baboon appendicular midshaft. **a)** example of a drifting osteon. The dark blue aspect represents the current proximal location of the osteon. The light orange aspect represents the "tail," showing the past drifting history across the cortex. **b)** example of a stationary (non-drifting) secondary osteon. **c)** example of typical remodeling in a histological section.

Table 1: Descriptive statistics of intracortical remodeling by appendicular site

Variable	**Humerus** (n=109)		**Femur** (n=76)		
	Mean	SD	Mean	SD	P
Age **(years)**	17	7.0	17	7.2	.989
On.Ar **(µm²,)**	6123.58	2115.69	8839.01	2497.53	.000*
H.Ar **(µm²,)**	602.11	95.16	642.88	101.81	.006*
DOn.Ar **(µm²,)**	9790.52	1712.63	8724.08	2127.85	.000*
Dh.Ar **(µm²,)**	814.61	116.99	650.48	92.08	.000*
OPD (#/**mm²)**	9.38	2.57	8.70	2.57	.073
%Po.Ar	24.64	10.86	20.94	8.09	.009*
%On.B	7.66	2.10	5.53	1.83	.000*
%C/T	69.13	5.39	69.76	5.15	.428
%C$_A$/T	52.13	8.83	55.19	7.19	.011*

Statistical difference between the sites was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 2: Descriptive statistics cross-sectional area measurements by appendicular site

Variable	**Humerus** (n=109)		**Femur** (n=76)		
	Mean	S.D	Mean	S.D.	P
Tt.Ar **(mm²)**	11.30	2.46	13.37	2.35	.001*
Ct.Ar **(mm²)**	7.78	1.68	9.30	1.61	.001*
Es.Ar **(mm²)**	3.52	1.08	4.07	1.09	.001*

Statistical difference between the sites was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 3: Descriptive statistics for intracortical remodeling dynamics by sex (Humerus)

Variable	**Females** (n=87)		**Males** (n=22)		
	Mean	SD	Mean	SD	P
Age **(years)**	18.06	7.0	14.43	6.16	.032*
Weight **(kg)**	16.97	4.38	28.27	7.21	.000
On.Ar **(µm²⁾**	5549.79	1668.64	8340.27	2280.89	.000*
H.Ar **(µm²⁾**	585.77	88.35	671.63	87.33	.000*
DOn.Ar **(µm²⁾**	9436.72	1581.22	11275.32	1560.47	.000*
Dh.Ar **(µm²⁾**	809.53	121.14	856.29	99.53	.227
OPD **(#/mm²)**	9.95	2.32	7.44	2.16	.000*
%Po.Ar	28.48	9.65	10.95	4.38	.001*
%On.B	7.94	2.27	6.94	1.91	.042*
%C/T	69.37	5.13	69.23	4.98	.621
% C$_A$/T	49.64	7.91	61.69	5.90	.000*

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 4: Descriptive statistics for intracortical remodeling dynamics by sex (Femur)

Variable	**Females** (n=49)		**Males** (n=27)		
	Mean	SD	Mean	SD	P
Age **(years)**	18.19	7.62	15.80	6.45	.173
Weight **(kg)**	17.02	3.92	28.17	6.68	.000
On.Ar **(µm²⁾**	9045.78	2635.13	8463.78	2224.16	.334
H.Ar **(µm²⁾**	677.30	73.59	580.42	116.69	.000*
DOn.Ar **(µm²⁾**	8670.28	2334.79	8821.73	1726.73	.769
Dh.Ar **(µm²⁾**	667.19	93.15	620.16	83.42	.026*
OPD **(#/mm²)**	8.86	1.77	8.41	3.63	.554
%Po.Ar	22.80	8.41	17.57	6.32	.007*
%On.B	5.60	1.79	5.40	1.91	.658
%C/T	69.62	5.44	70.01	4.67	.753
% C$_A$/T	53.79	7.45	57.72	6.04	.021*

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 5: Descriptive statistics cross-sectional area measurements (Humerus)

Variable	Females (n=87)		Males (n=22)		
	Mean	S.D	Mean	S.D.	P
Tt.Ar **(mm²)**	10.22	1.17	15.52	1.42	.000*
Ct.Ar **(mm²)**	7.05	.65	10.73	1.15	.000*
Es.Ar **(mm²)**	3.16	.80	4.79	.98	.000*

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 6: Descriptive statistics for cross-sectional area measurements (Femur)

Variable	Females (n=49)		Males (n=27)		
	Mean	S.D	Mean	S.D.	P
Tt.Ar **(mm²)**	11.97	1.21	15.91	1.67	.001*
Ct.Ar **(mm²)**	8.30	.74	11.11	1.08	.000*
Es.Ar **(mm²)**	3.67	.89	4.80	1.05	.000*

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 7: Results of stepwise regression analysis of intracortical remodeling dynamics (Humerus)

	OPD				H.Ar				On.Ar				DOn.Ar			
	Coefficient (SE)	B	P	R²	Coefficient (SE)	B	P	R²	Coefficient (SE)	B	P	R²	Coefficient (SE)	B	P	R²
N=109																
Sex	-2.532 (.549)	-.409	.000	.167	85.967 (21.160)	.367	.000	.127	2796.195 (433.189)	.531	.000	.282	1884.839 (367.035)	.446	.000	.199
Sex, Weight	-3.893 (.120)	.116	.006	.226												

Reported covariates are statistically significant at or below *p* value of 0.05

Results show significant **sex** effects on **OPD** (r^2=.167), **H.Ar** (r^2=.127), **On.Ar** (r^2=.282), and **DOn.Ar** (r^2=.199). **Sex and weight** modeled together accounted for slightly more variation (r^2=.226) in **OPD**.

Table 8: Results of stepwise regression analysis of intracortical remodeling dynamics (Humerus)

	%C/T				%Po.Ar				% C_A/T			
	Coefficient (SE)	B	P	R²	Coefficient (SE)	B	P	R²	Coefficient (SE)	B	P	R²
N=109												
Age	-.182 (.068)	-.253	.008	.064								
Sex					-17.690 (2.103)	-.633	.000	.400	12.260 (1.768)	.559	.000	.306
Sex, Age									11.343 (1.755)	.517	.000	.355

Reported covariates are statistically significant at or below p value of 0.05
Results show significant **sex** effects on **%Po.Ar** (r^2=.400), and **% C_A/T** (r^2=.306).
Sex and age modeled together account for slightly more variation in **% C_A/T** (r^2=.355).
%C/T is negatively affected by **age** (r^2=.064).

Table 9: Results of stepwise regression analysis of cross-sectional area measurements (Humerus)

	Tt.Ar				Ct.Ar				Es.Ar			
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
N=109												
Sex	5.286(.294)	.868	.000	.753	3.676(.187)	.886	.000	.783	1.610(.200)	.617	.000	.380
Sex, Age	5.499(.282)	.903	.000	.783					1.741(.194)	.667	.000	.442
Sex, Weight					2.967(.231)	.715	.000	.818				
Sex, Age, Weight	4.724(.374)	.776	.000	.801								

Reported covariates are statistically significant at or below *p* value of 0.05
Results show significant **sex** effects on all three variables, **Tt.Ar** (r^2=.753), **Ct.Ar** (r^2=.783), **Es.Ar** (r^2=.380). **Sex, age, and weight** modeled together account for slightly more variation in **Tt.Ar** (r^2=.801). **Sex and weight** modeled together account for slightly more variation in **Ct.Ar** (r^2=.818). **Sex and age** modeled together account for slightly more variation in **Es.Ar** (r^2=.442).

Table 10: Results of multiple regression analysis of intracortical remodeling dynamics (Femur)

	H.Ar				On.Ar				DH.Ar			
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
N=76												
Age					99.068 (38.193)	.289	.011	.083				
Sex	-96.833 (21.833)	-.458	.000	.210								
Weight									-3.170 (1.406)	-.254	.027	.064
Weight, Age									-3.305 (1.378)	-.264	.019	.116

Reported covariates are statistically significant at or below *p* value of 0.05
Results show significant **sex** effects in **H.Ar** (r^2=.210) and **age** effects in **On.Ar** (r^2=.083).
Weight alone significantly affects **Dh.Ar** (r^2=.064),
whereas **weight and age** modeled together accounts for approximately 50% more variation (r^2=.116).

Table 11: Results of stepwise regression analysis of intracortical remodeling dynamics (Femur)

| | %C/T | | | | %Po.Ar | | | | %C$_A$/T | | | |
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
N=76												
Age	-.247 (.077)	-.349	.002	.122					-.290 (.110)	-.293	.010	.086
Sex					-5.233 (1.856)	-.312	.006	.097				
Age, Weight									-.278 (.107)	-.281	.011	.151

Reported covariates are statistically significant at or below *p* value of 0.05
Results show significant **sex** effects in **%Po.Ar** (r^2=.097). **Age** effects are seen in **%C/T** (r^2=.122) and **%C$_A$/T** (r^2=.086). **Age and weight** modeled together account for slightly more variation in **%C$_A$/T** (r^2=.151).

Table 12: Results of stepwise regression analysis of cross-sectional area measurements (Femur)

	Tt.Ar				Ct.Ar				Es.Ar			
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
N=76												
Sex	3.939(.335)	.807	.000	.652	2.808(.211)	.840	.000	.706	1.130(.228)	.499	.000	.249
Sex, Age	4.120(.314)	.844	.000	.706					1.261(.212)	.557	.000	.379
Sex, Weight					2.004(.282)	.600	.000	.757				
Sex, Age, Weight	3.131(.070)	.642	.000	.740								

Reported covariates are statistically significant at or below *p* value of 0.05
Results show significant **sex** effects on all three variables, **Tt.Ar** (r^2=.652), **Ct.Ar** (r^2=.706), **Es.Ar** (r^2=.249). **Sex, age, and weight** modeled together account for slightly more variation in **Tt.Ar** (r^2=.740). **Sex and weight** modeled together accounts for slightly more variation in **Ct.Ar** (r^2=.757). **Sex and age** modeled together account for slightly more variation in **Es.Ar** (r^2=.379).
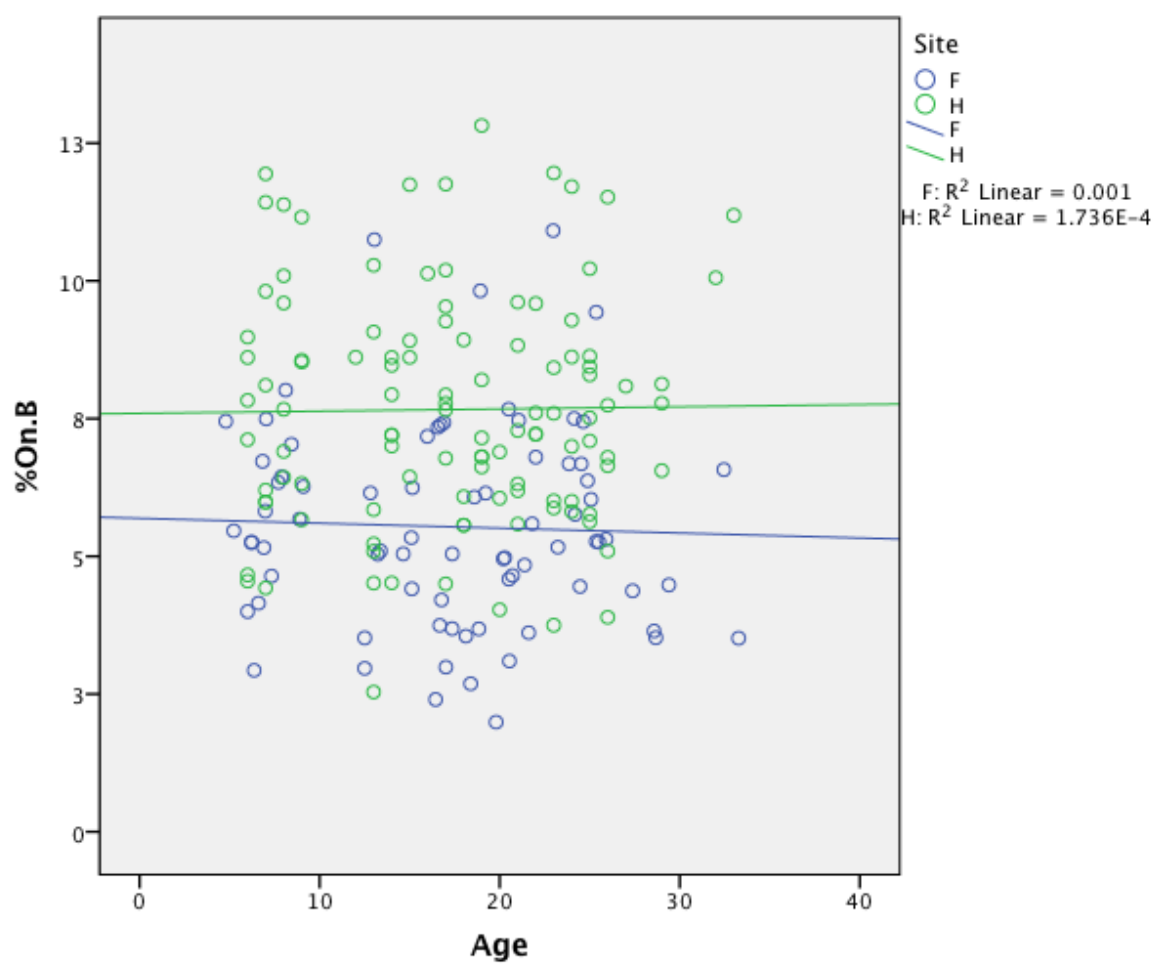
Fig. 2: Scatterplot indicating the significantly higher %On.B seen in the humerus (green) in contrast to the femur (blue).

Fig. 3: Scatterplot showing the significantly higher OPD in females (blue) over males (green) (Humerus)

Fig. 4: Scatterplot showing the significantly higher %Po.Ar in females (blue) over males (green) (Humerus)

Fig. 5: Scatterplot showing the significantly lower percentage cortical area adjusted for porosity (%C_A/T) seen in females (blue) over males (green) (Humerus)
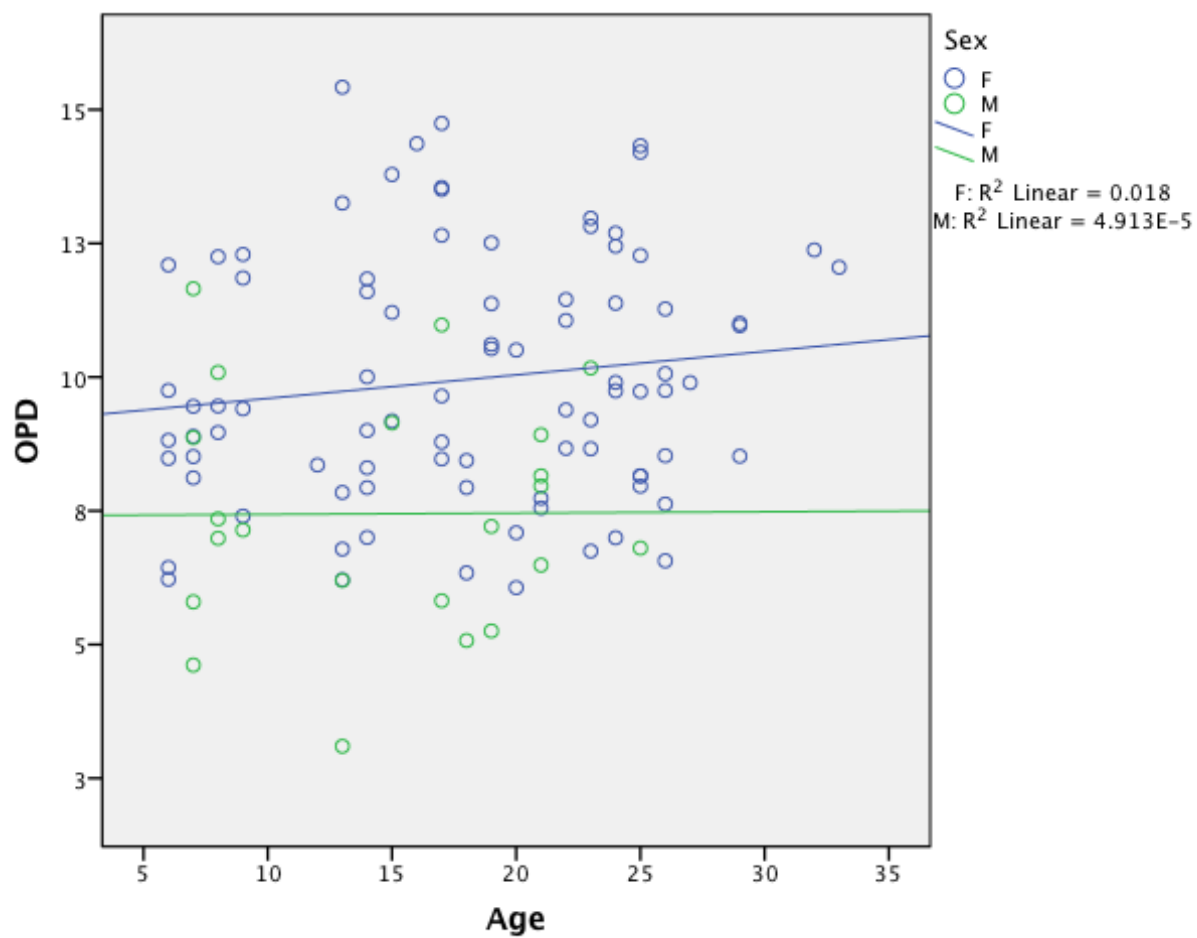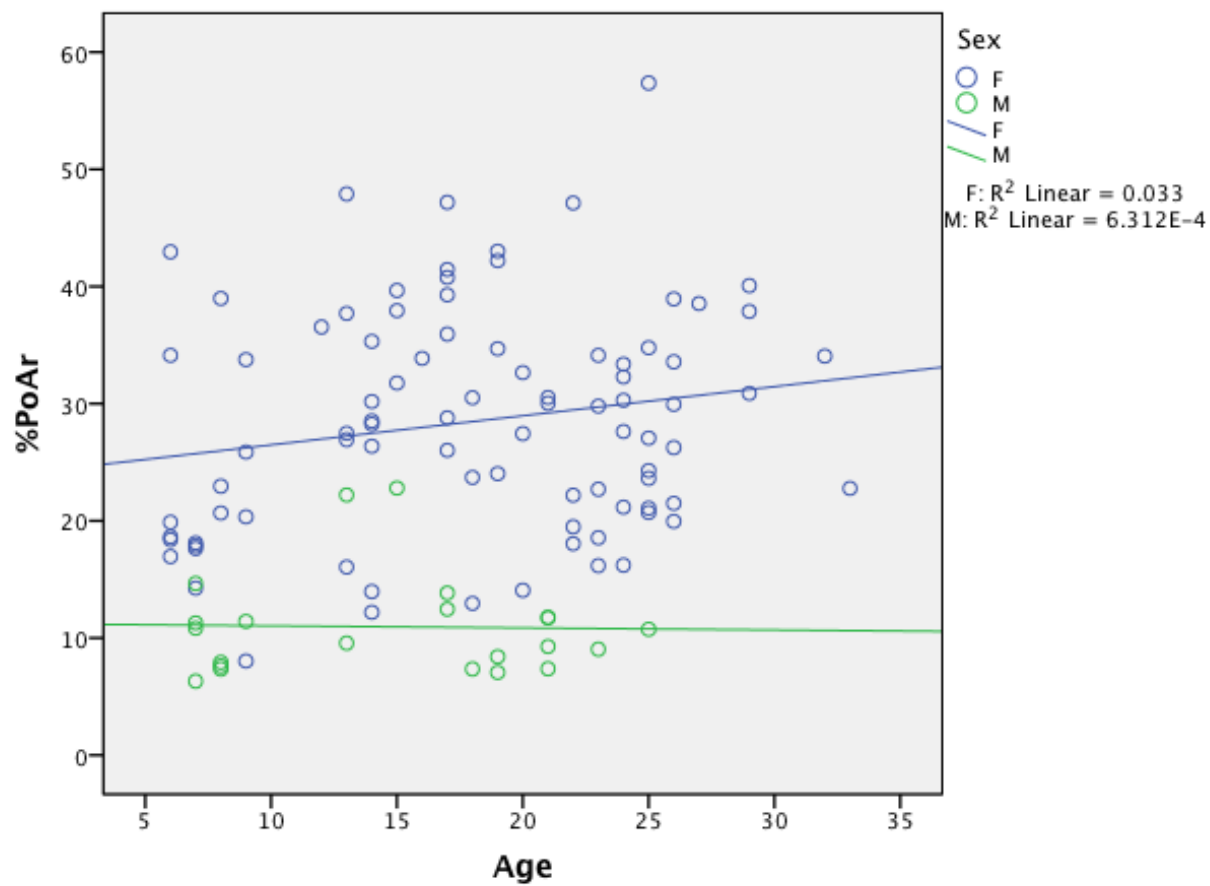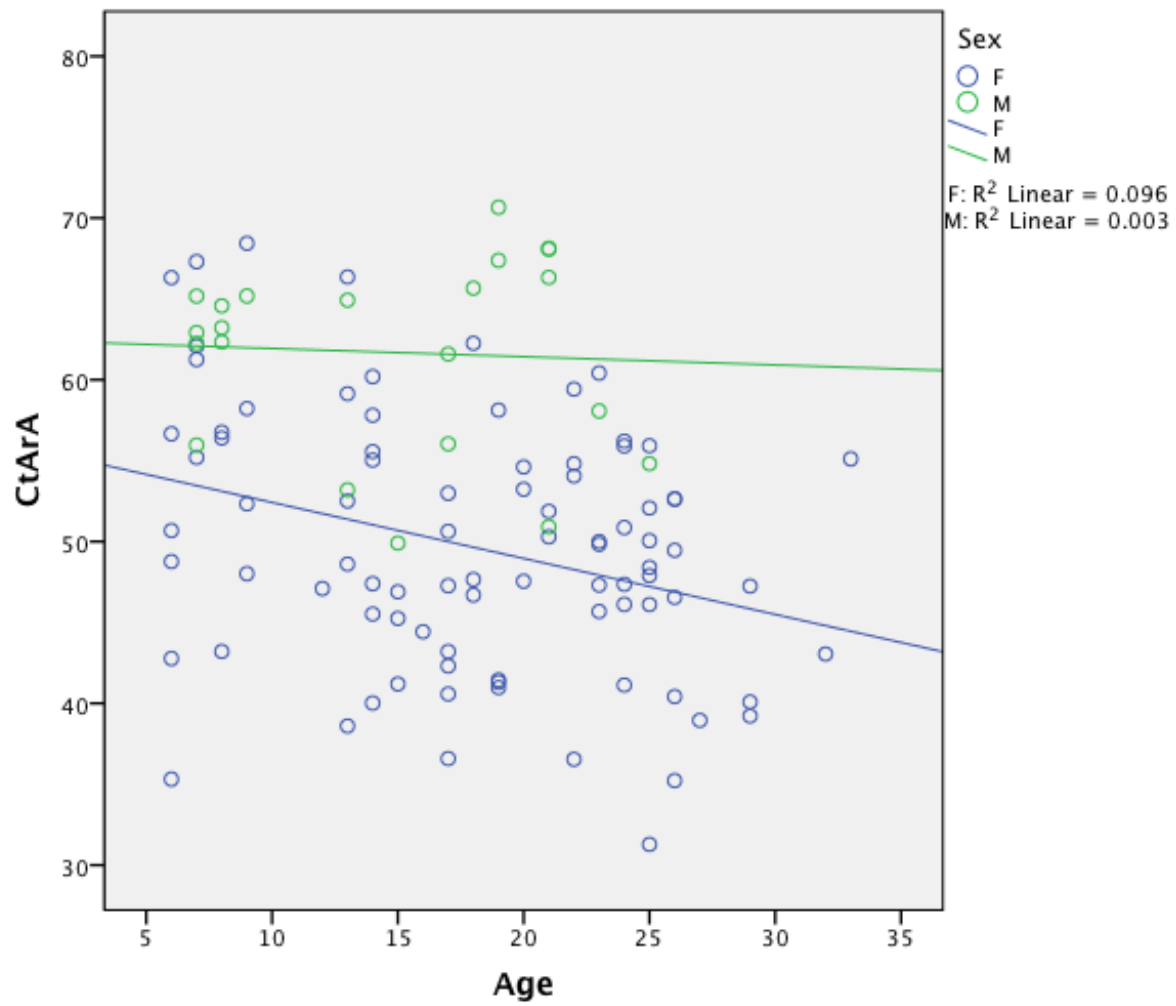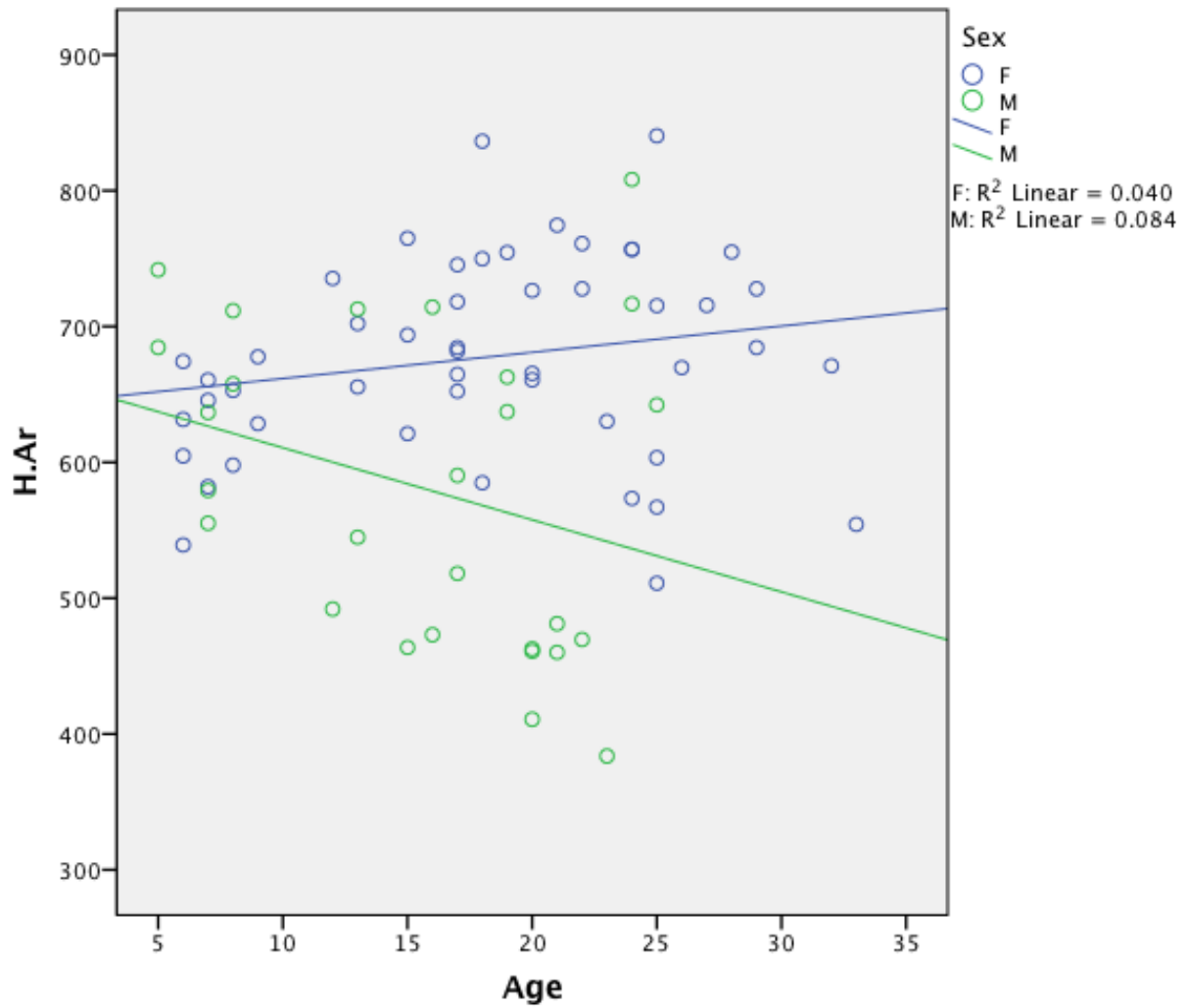
Fig. 6: Scatterplot showing the significantly larger H.Ar seen in females (blue) over males (green) (Humerus)
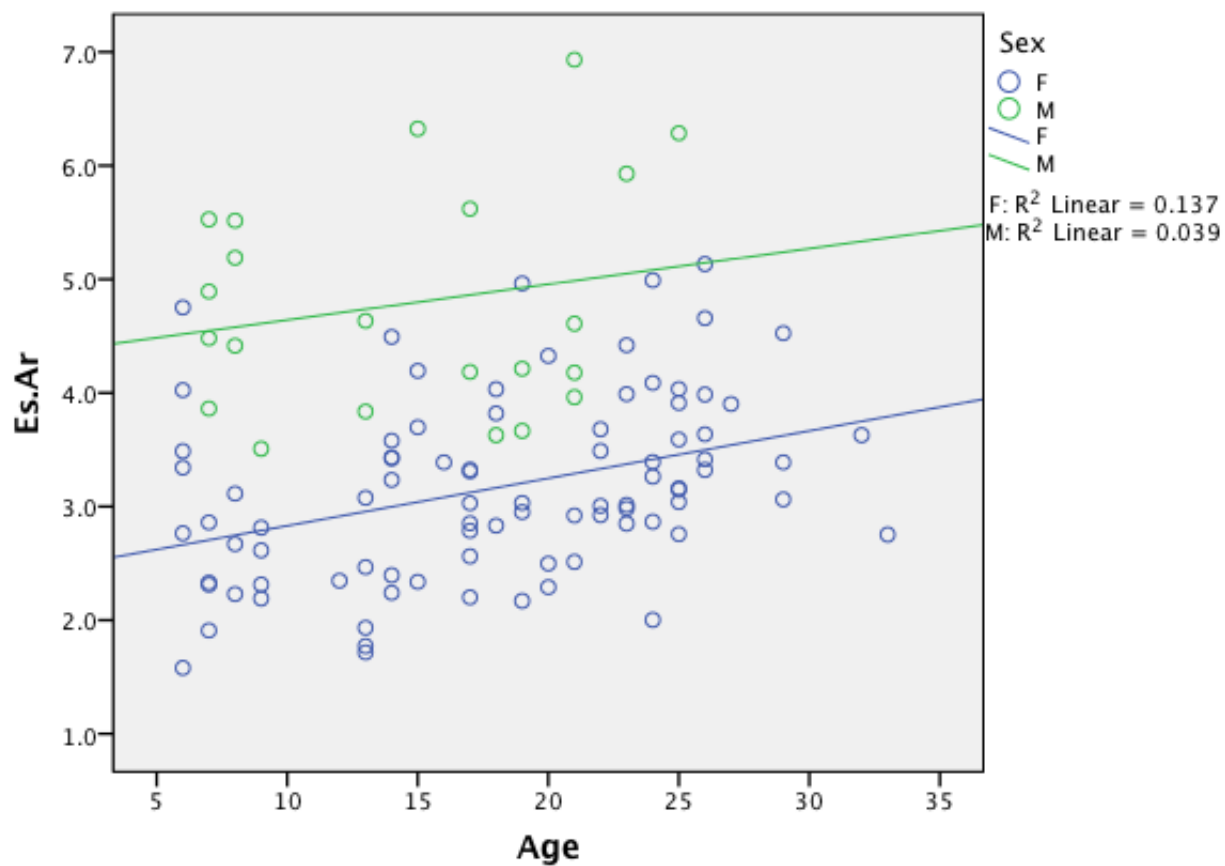
Fig. 7: Scatterplot showing the age and sex-related endosteal expansion (Es.Ar) (Humerus)

Fig. 13: Bivariate correlations of histomorphometric parameters (Humerus)- Total Sample

	%On.B	DOn.B	H.Ar	On.Ar	DOn.Ar	DH.Ar	%Po.Ar	%C_A/T	%C/T
OPD	.751**	.767**	-.250**	-.410**	-.302**	-.084	.452**	-.338**	.140
%On.B		.950**	-.078	-.127	.310**	.157	.276**	-.161	.181
DOn.B			-.182	-.327**	.185	.180	.405**	-.292**	.143
H.Ar				.614**	.256**	.210*	-.071	.141	.181
On.Ar					.365**	-.108	-.352**	.325**	.020
DOn.Ar						.480**	-.238*	.218*	.008
DH.Ar							.084	-.031	.113
%Po.Ar								-.901**	-.046
%C_A/T									.471**

Results show Pearson's Correlations values

* = Statistically significant at or below *p* value of 0.05

** = Statistically significant at or below *p* value of 0.01

Fig. 14: Bivariate correlations of histomorphometric parameters (Humerus)- Females

	%On.B	DOn.B	H.Ar	On.Ar	DOn.Ar	DH.Ar	%Po.Ar	%C_A/T	%C/T
OPD	.745**	.717**	-.025	-.197	-.097	.026	.317**	-.179	.187
%On.B		.976**	.026	-.077	.502**	.247*	.254*	-.112	.204
DOn.B			-.008	-.183	.475**	.297**	.282**	-.160	.161
H.Ar				.486**	.031	.104	.258*	-.122	.202
On.Ar					.024	-.33**	.027	-.007	.010
DOn.Ar						.474**	.044	-.021	.017
DH.Ar							.227*	-.152	.108
%Po.Ar								-.879**	-.052
%C_A/T									.517**

Results show Pearson's Correlations values

* = Statistically significant at or below *p* value of 0.05

** = Statistically significant at or below *p* value of 0.01

Fig. 15: Bivariate correlations of histomorphometric parameters (Humerus)- Males

	%On.B	DOn.B	H.Ar	On.Ar	DOn.Ar	DH.Ar	%Po.Ar	%C$_A$/T	%C/T
OPD	.804**	.863**	-.536*	-.442*	-.418	-.274	-.020	-.006	-.025
%On.B		.876**	-.206	.097	.115	-.139	-.188	.155	.073
DOn.B			-.418	-.221	-.121	-.079	-.006	.061	.072
H.Ar				.694**	.481*	.473*	-.085	.183	.188
On.Ar					.661	.122	-.437*	.296	.097
DOn.Ar						.405	.023	-.006	.001
DH.Ar							.403	-.079	.161
%Po.Ar								-.683**	-.241
%C$_A$/T									.874**

Results show Pearson's Correlations values

* = Statistically significant at or below *p* value of 0.05

** = Statistically significant at or below *p* value of 0.01

Fig. 16: Bivariate correlations of histomorphometric parameters (Femur)- Total Sample

	%On.B	DOn.B	H.Ar	On.Ar	DOn.Ar	DH.Ar	%Po.Ar	%C$_A$/T	%C/T
OPD	.628**	.629**	.487**	.171	-.433**	-.410**	.277*	-.225	-.026
%On.B		.995**	.253*	.459**	.337**	.041	-.013	.0181	.133
DOn.B			.240*	.452**	.334**	.027	-.013	.085	.139
H.Ar				.242*	-.252*	.193	.489**	-.304**	.112
On.Ar					.317**	.070	.195	-.181	.052
DOn.Ar				.		.603**	-.183	.195	.119
DH.Ar							.101	.105	.218
%Po.Ar								-.819**	-.104
%C$_A$/T									.653**

Results show Pearson's Correlations values

* = Statistically significant at or below *p* value of 0.05

** = Statistically significant at or below *p* value of 0.01

Fig. 17: Bivariate correlations of histomorphometric parameters (Femur)- Females

	%On.B	DOn.B	H.Ar	On.Ar	DOn.Ar	DH.Ar	%Po.Ar	%C$_A$/T	%C/T
OPD	.399**	.395**	.050	-.062	-.255	-.459**	.227	-.383**	-.355*
%On.B		.997**	-.061	.454**	.700**	.280	-.211	.145	-.003
DOn.B			-.074	.436**	.699**	.274	-.212	.149	.002
H.Ar				.206	-.083	.197	.399**	-.258	.060
On.Ar					.490**	.211	.092	-.137	-.111
DOn.Ar				.		.706**	-.192	.279	.253
DH.Ar							-.146	.365**	.455**
%Po.Ar								-.820**	-.107
%C$_A$/T									.654**

Results show Pearson's Correlations values

* = Statistically significant at or below *p* value of 0.05

** = Statistically significant at or below *p* value of 0.01

Fig. 18: Bivariate correlations of histomorphometric parameters (Femur)- Males

	%On.B	DOn.B	H.Ar	On.Ar	DOn.Ar	DH.Ar	%Po.Ar	%C$_A$/T	%C/T
OPD	.882**	.869**	.773**	.436*	-.774**	-.510**	.387*	-.068	.321
%On.B		.995**	.618**	.472*	-.490**	-.455*	.383*	.000	.415*
DOn.B			.607**	.497**	-.453*	-.442*	.394*	-.012	.409*
H.Ar				.254	-.602**	-.033	.497**	-.178	.277
On.Ar					-.168	-.363	.399*	-.214	.114
DOn.Ar				.		.431*	-.144	-.071	-.273
DH.Ar							.130	-.284	-.293
%Po.Ar								-.758**	-.070
%C$_A$/T									.704**

Results show Pearson's Correlations values

* = Statistically significant at or below *p* value of 0.05

** = Statistically significant at or below *p* value of 0.01

Chapter 5

Effect of reproduction on intracortical remodeling and cross-sectional area dynamics
_____

Introduction

      As discussed in Chapter Two, pregnancy and lactation are periods of increased maternal skeletal turnover and result in significant temporary reductions to bone mass density (BMD). In humans these reductions in bone mass are typically reversed following infant weaning and resumption of maternal menses (Kalkwarf and Specker, 1995; Kolthoff et al., 1998; Lopez et al., 1996; Sowers et al., 1993). However, BMD measurements alone do not offer insight into the qualitative changes that occur with increased bone turnover, and the cumulative effects of reproduction on altering cortical bone microstructure are still not well understood. Remodeling typically affects the quantity of bone, but not quality (e.g. Young's modulus and tensile strength) (Woo et al., 1981). Within individual BMUs, remodeling cycles aid in the removal of old, weaker lamellae, replacing it with freshly mineralized bone (Gourion-Arsiquaud et al., 2009). However, if osteoclastic activity dominates enough to result in significant intracortical porosity, this is associated with dramatic decreases in fracture toughness (Yeni et al., 1997), resistance to fracture (Ebacher et al., 2007), and compressive strength (Evans and Vincentelli, 1974; McCalden et al., 1994). Currently, it is unclear how the hormonal fluctuations and increased calcium demands associated with pregnancy and lactation affect aspects of cortical bone microstructure, and further, if these alterations have long-term effects on skeletal health and fragility. Retrospective studies of reproductive histories and cancellous BMD measurements in pre and post-menopausal women have produced ambiguous results with some showing negative (Henderson et al., 2000; Durson et al., 2006; Demir et al., 2008; Hopkinson et al., 2000; Okyay et al., 2013), neutral (Chowdhury et al., 2002; Lenora et al., 2009; Kojima et al., 2002), or positive associations (Canal-Macias et al., 2012; Schnatz et al., 2010), and even less is known about the long-term effects on cortical bone.

      Bone remodeling in human women during and after the gestational and lactation periods has rarely been directly evaluated. Direct study of the microstructure of cortical bone would require invasive surgical procedures to remove bone core samples and would put the mother and fetus at an unnecessary risk. Instead, biochemical markers of bone resorption and deposition are taken from maternal blood and urine samples to offer an indirect, non-invasive view of remodeling. These data offer important insights into the general, whole-skeleton fluctuations in bone formation and resorption over pregnancy and lactation, but they cannot relay information about the specific changes to the microstructure. Animal models have been essential in furthering an understanding of the cortical cross-sectional area and turnover changes that occur during reproduction. In rats, pregnancy is associated with increased periosteal modeling and relatively little intracortical turnover (Miller and Bowman, 2004). Changes with lactation are more significant, including large reductions in femur cortical area and increases in porosity (Liu et al. 2012), as well as increased osteoclastic activity (Miller and Bowman, 2004). However, specific changes to microstructure (e.g. osteon sizes and OPD) cannot be examined in rats

because they do not organize bone into Haversian systems (Jee and Yao, 2001; Nelson et al., 1982; Felicio et al., 1984) like humans. Larger-bodied mammals (e.g. dogs, monkeys) do have a Haversian system of bone organization similar to humans, and are more appropriate models of intracortical turnover and microstructural change. Dynamic histomorphometric studies of cortical bone change revealed an increase in the total number of osteons as a result of increased BMU activation during lactation in dogs (Vadja et al., 1999), and an increased bone formation rate in lactating macaques (Lees and Jerome, 1998). However, a thorough retrospective investigation of the long-standing changes to intracortical Haversian remodeling through characterizations of osteon population density, percent osteonal bone, Haversian canal size, and osteon areas with respect to parity and lactation histories have not been characterized in any animal model (dog, rat, or old world monkey).

Investigation of bone micro-morphological variation in the adult skeletons of large bodied cercopithecine primates like *Papio hamadryas* with complete individual reproductive histories could lend considerable clarity into how variation in parities, lactation lengths and interbirth intervals affect variation in skeletal micro-organization. Female baboons and humans share many cercopithecine traits, including a large body size, relatively long lifespan, similar reproductive physiology and endocrinology (Brommage, 2001), and similar menstrual cycle timing and phases of hormonal fluctuations (Chen et al., 1998; Martin et al., 2003). Skeletally, baboons and humans also have the same organization of bone microarchitecture in Haversian systems (Jerome and Peterson, 2001), as well as the same patterns of bone degeneration during aging (Aufdemorte et al., 1993; Cerroni et al., 2000; DeRousseau, 1985; Havill 2003a; Kammerer et al., 1995). Short-term skeletal modifications during pregnancy and lactation in baboons also parallel those seen in humans, showing little bone mass change during pregnancy until the third trimester (Lees et al., 1998), significant bone loss during lactation (Ott et al., 1999), followed by a period of increasing bone mass (recovery) after weaning and resumption of menses (Lees et al., 1998). Baboons also exhibit variation in parity (number of offspring produced over the lifetime), interbirth interval (the duration between successive pregnancies) and in lactation lengths with some female baboons weaning at six months and others allowing suckling for up to a year (Dr. Linda Brent, personal communication). Studies of reproductive history and bone health in baboons and macaques have been for the most part limited to quantitative BMD measurements in skeletal sites with a high proportion of trabecular bone (Cerroni et al., 2003; Havill et al., 2008). One study (Bowden et al., 1979) directly assessed cortical bone health in relation to reproductive history. Bowden et al., (1979) observed that that higher parity (>3) is associated with significantly increased metacarpal thickness in captive macaques (*Macaca fascicularis* and *M. nemestrina*).

Hypotheses

Using bone samples from captive baboons from the SNPRC with known reproductive histories, the present study builds upon the patterns previously observed (Bowden et al., 1979; Cerroni et al., 2003; Havill et al., 2008) and further clarifies how cortical bone micro and macrostructure is influenced by differences in parity, interbirth interval, and lactation duration. Here, bone quality and quantity is assessed through static histormorphometry at two appendicular sites (midshaft humerus and femur). Aspects of cortical remodeling (e.g. osteon area and OPD) are examined in conjunction with cross-sectional area (e.g. cortical area and total

area), and this offers a comprehensive view of variation in both modeling and remodeling. The central working hypothesis of this study is that reproductive history is a significant factor in generating bone microstructural variation in the adult female baboon. A secondary hypothesis is that high offspring production and care, through increased parity and long lactation durations, will have a cumulative effect of increased resorption over deposition and will have negative effects on overall bone health. A full range of adult ages (6-33 years) is assessed, which allows this work to address how reproductive history affects bone variation throughout the entire aging process in the baboon. As the long-term intracortical organizing effects of reproduction over the aging process have not yet been evaluated in the literature, this work is essential in advancing our understanding of the long-term effects of reproduction on cortical bone quality.

Methods

The midshaft humerus and femur histological slides used in Chapter 4 are further analyzed statistically in this study. In this study only female animals were included, with n=87 (humerus), and n=49 (femur). Details of data collection, materials and methods are reviewed in Chapter 3 and Chapter 4. The microstructural and cross-sectional parameters analyzed here are also reviewed in detail in Chapter 4 and are summarized in Chapter 3.

1. **Osteon area** (μm^2, On.Ar): The total area circumscribed by the reversal (cement) line averaged across all osteons within the field of assessment.

2. **Haversian** (central) **canal area** (μm^2, H.Ar): The total area of the Haversian canal averaged across all canals within the field of assessment.

3. **Osteon population density** (#/mm^2, OPD), (osteon number + osteon fragment number)/bone area: The number of secondary osteons with intact Haversian canals plus the number of osteon fragments (the number of secondary osteons in the field of view that are without intact Haversian canals) normalized by the total bone tissue assessed.

4. **Drifter osteon area** (μm^2, DOn.Ar): The total area circumscribed by the most recent/proximal reversal (cement) line for each drifter osteon, averaged across all drifter subtype osteons within the field of assessment.

5. **Drifter osteon Haversian canal area** (μm^2, Dh.Ar): The total area of the drifter osteon Haversians canal averaged across all drifter osteon canals within the field of assessment.

6. **Percent osteonal bone** (%On.B), (osteonal area/bone area) x 100: The proportion of the observed cortex occupied by secondary osteons with intact Haversian canals

7. **Percent porosity area** (%Po.Ar) (total void areas including Volksman's canals, Haversian canals, immature osteons with unfilled portions, and other pores/ bone area) x100: The ratio of intracortical void areas (including central, longitudinal

canals and other porous spaces but excluding osteocyte lacunae) to total assessed bone area.

8. **Percent cortical area** (%C/T) (Ct.Ar/Tt.Ar x 100): cortical area relative to the total area, expressed as a percentage.

9. **Percent absolute cortical area** (%C_A/T) (Ct.Ar$_A$/Tt.Ar x 100; Ct.Ar$_A$= Ct.Ar-Po.Ar in mm^2): absolute cortical area (taking into account porosity) relative to the total area, expressed as a percentage.

Macroscopic measurements of the midshaft humerus and femur include:

1. **Total area** (mm^2, Tt.Ar): the total area of the bone midshaft, including cortical and endosteal components: The outer margin (periosteal border of the cortice) is traced and the Bioquant software determines the total area for the midshaft specimen.

2. **Cortical area** (mm^2, Ct.Ar) (Tt.Ar-Es.Ar): cross-sectional area of cortical bone between periosteal and endosteal surfaces. Cortical area is manually calculated by subtracting the total area from the endosteal area.

3. **Endosteal area** (mm^2, Es.Ar): area of the marrow cavity. The inner margin (endosteal border) is traced and the Bioquant software determines the endosteal area for the midshaft specimen).

Reproductive history:

Several aspects of female baboon reproductive history are recorded at the SNPRC and the calculations are detailed in Chapter 3. For this study, **Parity**, mean interbirth interval (**IBIavg**), percent of adult life spent nursing an infant (**%LifeLact**) were collected from extensive clinical records available for each animal and are summarized below:

1. **Parity** (number of live offspring; #): Total offspring number born to a dam

2. **%LifeLact** (percentage of life spent lactating; %): Total percentage of dam's life spent lactating.

3. **IBIavg** (average interbirth interval; days): Average time between successive births of live offspring for a dam

<u>Statistical analysis</u>

Normality tests (Shapiro-Wilk's test, skewness and kurtosis) were assessed first to determine if parametric analyses were appropriate for this sample and subsample. Parametric bivariate correlations were then performed in order to assess the relatedness of reproductive variable to each other and with age (**Table 1**). Exploratory statistics were carried out to assess ranges and means for reproductive variables in two age cohorts: young adults (aged 6-18 years) and old adults (aged 19-33 years) and are summarized in **Tables 2** and **3**. The mean age of onset of irregular monthly cycling and peri-menopausal phenomena is reported to be 19.0 years in SNPRC baboons (Martin et al., 2003), therefore females were grouped into two separate reproductive cohorts for analysis: a normally cycling, young adult group, and an irregularly cycling, older adult group (similar to a pre-menopausal cohort). In humans, it is standard practice to assess regularly cycling young adults separate from pre-menopausal adults as there are significant hormonal differences between the two groups (e.g. Madimenos et al., 2012) and a recent study by Macrini et al., (2013) found that female captive baboon hormonal cycling status was significantly associated with skeletal osteoarthritis changes. Descriptive statistics and t-tests were performed to show differences in remodeling variables and cross-sectional area measurements by site (midshaft humerus and femur) (**Tables 4-5**).

Stepwise multiple linear regression analyses were performed to investigate the relationships between cortical bone variables (e.g. H.Ar, Ct.Ar), age, body weight, and reproductive history variables (**Tables 6-12**). Microstructural and cross-sectional area parameters were entered as dependent variables in regression models and age, body weight, parity, IBIavg, and %LifeLact were used as the independent variables. For each analysis, all females (per site sample) were analyzed as a whole group first to observe the overall effect across all ages, and then assessed separately into age cohorts to account for the relative effects of age and hormonal differences. One-way ANCOVAs (with age held constant) were examined in the more robust humerus groups but not the femur groups due to small sample sizes (i.e. <10 per group). The purpose of these ANCOVAs was to elucidate if high vs. low parity, high vs. low %LifeLact differed significantly in any of the cortical bone parameters (**Tables 13-16**). Tukey post-hoc tests revealed which groups within an ANCOVA were statistically different. All statistical analyses were performed using SPSS 21.0 (SPSS).

Results

<u>Sample characteristics:</u>

A full spectrum of adult ages is well represented in both samples (**Fig. 1, Fig. 2**) and the average age (approx. 18 years) does not differ significantly between sites (**Table 4**). Body weight does not differ significantly between the humerus and femur samples (**Table 4**). Reproductive profiles by site and by age cohort are summarized in **Tables 2-3**. Age, parity, IBIavg and %LifeLact are significantly different between young and old adult cohorts in the humerus and weight does not differ significantly (**Table 2**). Age, parity, and IBIavg differ significantly between young and old adult cohorts in the femur; weight and %Lifelact are not significantly different (**Table 3**). Bivariate correlations show that age is significantly correlated with all

aspects of reproductive history; as age increases, so does parity, %LifeLact, and IBIavg (**Table 1**), as expected.

Site differences on intracortical remodeling:

Significant site differences here are similar to the differences seen in Chapter 4, where males and females were analyzed together. In general, osteon area and Haversian area (On.Ar, H.Ar) are significantly larger in the femur, and drifting osteon and Haversian areas (DOn.Ar, DH.Ar) are significantly larger in the humerus (**Table 4**), as was seen in Chapter 4. Also similar is the significantly higher porosity (%Po.Ar), percent osteonal bone (%On.B), and lower adjusted cortical area (%C_A/T) of the humerus relative to the femur (**Table 4**). Cross-sectional geometry differs significantly between the two sites with the femur showing larger areas in all parameters: Tt.Ar, Ct.Ar, and Es.Ar (**Table 5**). This is unsurprising considering the large length and robusticity differences between the hind and forelimbs.

*Effects of **age** on intracortical remodeling:*

Age effects are also similar to those seen in Chapter 4. However, some differences in percent variation explained are seen because only females are analyzed here, narrowing the range of means and lowering the sample size. The result of this is a better fit with the age regression slopes, and thus larger r^2 values than were seen in Chapter 4. For example, in the femur, age explains roughly 30% of the variation seen in Dh.Ar (p=.001)(**Table 9**) and age combined with weight explains roughly 41%(p=.001)(**Table 9**). Overall, the relationship of age, and age and weight to Dh.Ar are similar to the results in Chapter 4, but further amplified now that male means are removed from the regression model. Here in the femur, age explains 18% of variation in %C/T (as opposed to 12% in Chapter 4), and 16% of variation in %C_A/T (as opposed to 8% in Chapter 4) in the young adult age cohort (**Table 10**). Age does not appear to have a strong effect on these variables in the old age cohort. In the midshaft humerus, age is not a significant predictor of bone remodeling parameters when reproductive variables are also considered in stepwise regression models. The strong age effects in the femur may be related to the difference in samples sizes between the femur and humerus (*femur* n=49; *humerus* n=87).

*Effects of **weight** on intracortical remodeling:*

As seen in Chapter 4, body weight demonstrates few significant effects in the absence of other variables (e.g. age, %LifeLact). At the midshaft humerus, weight has a weak but positive relationship with osteon population density (OPD) (r^2=.071, p=.027) (**Table 6**). In the femur, weight has a negative effect on DOn.Ar in the old age group only (r^2=.226, p=.046) (**Table 9**).

*Effects of **Parity** on intracortical remodeling*:

Stepwise regression models show that parity exhibits a strong negative influence on %C/T (r^2=.183, p=.001) (**Table 7**) at the midshaft humerus (**Fig. 3**) in both the all adults group and old age cohort (r^2=.193, p=.008) (**Table 7**) (**Fig. 2**). The R^2 values do not differ significantly between the whole group and the old age cohort, suggesting that parity has little effect on this variable in younger animals and only exhibits a strong influence after the age of 19 years. At the midshaft humerus, an ANCOVA analysis (age held constant) with post-hoc comparisons between nulliparous, low, and high parity groups found significant differences between low and high parity groups in %C/T (**Table 14**). This ANCOVA clarifies that the negative relationship with %C/T is most clearly seen in the transition from low parity (μ 71.07) to high parity (μ 66.48). In %C/T the means for in the nulliparous and low parity groups are not significantly different, but are both larger than in the high parity group, suggesting a neutral effect of low parity, but negative effect once the offspring number was greater than 9. Interestingly, the ANCOVA also shows significant differences between low and high parity groups in drifter osteon areas (DOn.Ar), with the low parity group showing a lower DOn.Ar (μ 8934) (**Table 12**) than the high parity group (μ 1037) (**Table 12**). High parity is associated with significantly larger drifter osteon areas. Overall, at the midshaft humerus, the influence of high parity is to decrease the proportion of cortical area relative to total area, and to increase the drifter osteon area.

*Effect of **IBIavg** on intracortical remodeling:*

Regression models show that IBIavg explains little overall variation in cortical bone variables in the humerus and femur. At the midshaft humerus, IBIavg is associated with a small but significant decrease in %Po.Ar (r^2=.080, p=.035)(**Table 6**). At the midshaft femur, IBIavg shows a positive relationship with On.Ar (r^2=.156, p=.031)(**Table 9**) in the whole group, suggesting that longer IBIs increase osteon size at this site.

*Effect of **%LifeLact** on intracortical remodeling:*

An ANCOVA analysis (age held constant) with post-hoc comparisons between 0%, low, and high lactation groups showed significant differences in %Po.Ar between low lactation (μ 23.15)(**Table 14**) and high lactation (μ 33.02) (**Table 14**) groups at the midshaft humerus. The low %LifeLact group (μ 53.53)(**Table 15**) and high %LifeLact group (μ 46.91)(**Table 15**) also differed significantly in %C_A/T (**Fig. 3**), with %C_A/T reduced as a result of high parity. Overall the effect of high %LifeLact at the humerus is to increase %porosity and to lower the amount of cortical bone**.** The latter is an unsurprising result since high porosity is negatively associated with %C_A/T values (see Chapter 4 for a review). Stepwise regressions also confirmed that %LifeLact is associated with reduced %C_A/T primarily in the old age group (r^2=.179, p=.011) (**Table 7**), and the decrease is observed in a scatterplot as well (**Fig 4**).

Cross-sectional area:

In the humerus, %LifeLact is positively associated with total area (Tt.Ar) when all ages are considered (r^2=.117, p=.006) (**Table 8**). The effect of %LifeLact is further amplified in the old adult group where it explains roughly 29% of variation (p=.001)(Table 8). Weight and %LifeLact together explain nearly half of variation in Tt.Ar in old age (r^2=.420, p=.000)(**Table 8**). An ANCOVA analysis (age held constant) with post-hoc comparisons between 0%, low, and high lactation groups showed significant differences in Tt.Ar between 0% lactation (μ 9.86) and high lactation (10.61) groups (**Table 15**). Interestingly, the ANCOVA found significant differences in Tt.Ar between nulliparous (μ 9.81) and high parity (μ 10.76) as well as low parity (μ 9.848) and high parity (μ 10.76) groups (**Table 15**). Overall, both high parity and high %LifeLact are associated with significantly increased total area (**Tables 13, 15**). The effects of %LifeLact and weight on Tt.Ar are similar in the femur (**Table 11**), where weight accounts for roughly 19% of variation (p=.015) (**Table 11**), and weight and %LifeLact together account for roughly 34% (p=.007) (**Table 11**) in the all adult group. The effects of %LifeLact and weight at the femur are also positive, increasing total area.

In the humerus, cortical area (Ct.Ar) is significantly explained by weight alone (r^2=.181, p=.001) (**Table 8**) and in combination with %LifeLact (r^2=.289, p=.000)(**Table 8**). The combination of %LifeLact and weight explains slightly more Ct.Ar variation in the old adult group (r^2=.381, p=.000) (**Table 8**). An ANCOVA analysis (age held constant) with post-hoc comparisons between 0%, low, and high lactation groups showed significant differences in Ct.Ar between 0% lactation (μ 6.87) (Table 16) and high lactation (μ 7.38) groups. The overall effect of %LifeLact is positive with cortical area in the humerus. Similar effects are seen in the femur, where Ct.Ar is significantly explained by weight (r^2=316, p=.001) (**Table 11**) in the all adult group as well as in the young adult group (r^2=.504, p=.010) (**Table 11**). Reproductive history is not a significant predictor of Ct.Ar in the femur, and this could be a result of smaller sample size, or locomotor differences between the hind and forelimb.

In the humerus, endosteal area (Es.Ar) is significantly explained by parity (r^2= .185, p=.001)(**Table 8**) when all ages are considered. An ANCOVA analysis (age held constant) with post-hoc comparisons between 0, low, and high parity groups showed significant differences in Es.Ar between nulliparous (μ 3.04) and low parity (μ 2.87) groups as well as nulliparous and high parity (μ 3.67) groups (**Table 13**). High parity is associated with significantly larger medullary spaces in the humerus. In the femur, Es.Ar is explained by %LifeLact (r^2=.336, p=.048) (**Table 11**) in the young adult group as well as old adult group (r^2=.265, p=.029)(**Table 11**).

Discussion

The majority of animal studies investigating micro-architectural changes with pregnancy and lactation have focused on trabecular bone sites (*rats* Tojo et al., 1998; Bowman et al., 2002; Vadja et al., 2001; Liu et al., 2012; *dogs* Miller et al., 1989; Fukuda and Iida, 1993; *macaques* Lees and Jerome, 1998; Ott et al., 1999). Intracortical remodeling changes have been less well characterized in the literature; however, some overall trends are seen. In both dogs (Vadja et al., 1999) and rats (Liu et al., 2012; Vadja et al., 2001; Miller and Bowman, 2004), there is an increase in cortical bone cellular activity and turnover during lactation. In dogs, BMU activation and number of osteons are increased during lactation (Vadja et al., 1999) and in rats, osteoclast activity (Miller and Bowman, 2004) and porosity (Miller and Bowman, 2004; Liu et al., 2012) are significantly increased during this period. The few studies that do focus on changes in cortical bone deposition and resorption during pregnancy and lactation do so through dynamic histomorphometry where osteoid (undermineralized bone tissue), osteoclasts and osteoblast activity can be detected (Vajda et al., 1999; Miller and Bowman, 2004). The present study is the first to assess Haversian remodeling microstructural changes with respect to reproductive history.

At the midshaft humerus, the overall effect of parity is to increase DOn.Ar size. .An ANCOVA analysis (age held constant) shows significant differences between low and high parity groups in drifter osteon areas (DOn.Ar), with the low parity group showing a lower DOn.Ar value ($\mu= 8934$) (**Table 12**) than the high parity group ($\mu= 1037$) (**Table 12**). At the midshaft femur, IBIavg shows a positive relationship with On.Ar ($r^2 =.156$, p=.031) (**Table 9**) in the whole group, suggesting that longer IBIavgs increase osteon size at this site. Overall, in baboons, reproductive history is associated with increases in osteon sizes at both the humerus and femur. These changes are likely related to the significantly elevated levels of estrogen seen during pregnancy in macaques (Hein et al., 1989) and baboons (Hendrickx and Dukelow, 1995). Elevated estrogen is associated with increases in osteoblastic activity and decreases in osteoclastic activity. For example, a study of bone markers in cynomolgus macaques during their menstrual cycle demonstrated decreased bone resorption and osteoclast activity during the peak estrogen phase (Hotchkiss and Brommage, 2000). During pregnancy in cynomolgus macaques, serum alkaline phosphatase (ALP), and bone gla-protein (BGP) also indicated low rates in bone turnover and resorption (Lees and Jerome, 1998). It is likely that the increased osteoblast and reduced osteoclast activity could result in larger osteons, with a significant cumulative effect after multiple gestational cycles that occur with high parity, as seen here.

At the midshaft humerus, significant differences in %Po.Ar were seen between low (μ 23.15) and high (μ 33.02) %LifeLact groups (**Table 14**). Significantly increased appendicular cortical porosity has also been observed experimentally in lactating mice (Liu et al., 2012). A 36% increase in porosity was observed in lactating mice over non-lactating controls (Liu et al., 2012). After a subsequent period of recovery (28 days post-weaning), porosity was reduced, but still 16% higher than in non-reproducing controls. This suggests that adequate periods of recovery between subsequent offspring are necessary to repair the high porosity accumulated during lactation, but long-standing changes in porosity may persist. In another mouse study, as early as two weeks post-weaning significant active mineralization was present on eroded surfaces of cortical endosteum (Miller and Bowman, 2004), indicating a skeletal transition from resorption (during lactation) to formation (after weaning). In the present study, some evidence

for this in baboons is also shown. Regression analyses showed that increased IBIavg is associated with a small but significant decrease in %Po.Ar (r^2=.080, p=.035) (**Table 6**) at the midshaft humerus. Therefore, although the overall effect of high %LifeLact increases %Po.Ar in baboons, some positive buffering of longer IBIavgs is seen.

Significant effects of reproductive history are also seen in cross-sectional area at the humerus and femur. At the midshaft humerus, significantly increased Tt.Ar is seen in both the high %LifeLact group (**Table 15**) and high parity group (**Table 13**). Ct.Ar is also significantly increased in the high lactation group (**Table 15**). Stepwise regressions show that weight and %LifeLact together account for a large percentage of variation in both Tt.Ar (42% in the *humerus*, **Table 8;** 34% in the *femur* **Table 11**) and Ct.Ar (38% in the *humerus*, **Table 8**) in the old adult age cohort (ages 19-33). Together, the larger Tt.Ar and Ct.Ar in females with high %LifeLact and parity indicate significant periosteal apposition in these groups. The increased biomechanical strain associated with carrying suckling infants, in addition to the increased maternal body weight during pregnancy, may have stimulatory anabolic effects on the periosteum of cortical bone and may account for the increased total and cortical area measurements seen in these groups. Additional support for this hypothesis is found in Bowden et al. (1979) where significant increases in metacarpal thickness are seen in age-matched macaque females with high parity. Although age-related loss in cortical thickness is seen in macaques, females with high parity had significantly increased cortical area (Bowden et al., 1979). A similar result was seen by Specker and Binkley, (2005) where Hutterite women with high parity (>7 children) showed a 4% increase in cortical thickness at the femoral neck. However, measurements of cortical area thickness do not reveal information about the porosity of intracortical bone, and "true" measurements of cortical area may be significantly lowered after accounting for the percentage of void areas inside the cortex. In this study, although the Tt.Ar and Ct.Ar are larger with increased %LifeLact, the total percentage of adjusted cortical area (%C_A/T) after accounting for the porosity is lower in these females (**Fig. 4**). This suggests that the behavioral aspects of infant-rearing associated with higher %LifeLact increase the outer cortex (periosteum) of the humerus but low estrogen levels and increased osteoclast activity during breastfeeding decrease the total amount of endosteal bone through increased porosity. This pattern has also been observed in rats, where significant increases in periosteal deposition and cross-sectional area were seen during pregnancy (Miller et al., 1986; Vadja et al., 2001), followed by significant resorption of the endosteum during lactation (Miller and Bowman, 2004). Miller and Bowman, (2004) hypothesize that the inverse relationship of periosteal deposition with endosteal resorption may have an adaptive mechanical significance. Endocortical resorption results in a decrease in mechanical strength of cortical bone (Currey and Hughes, 1973; Vajda et al., 2001) and the increases in periosteal apposition and overall cross-sectional area may help protect the maternal skeleton from fracture during this catabolic period (Miller and Bowman, 2004). Lactation-induced increased endocortical resorption without negative changes to cross-sectional geometry has also been observed in humans. Laskey et al., (2011) observed significant BMD decreases in femoral shaft cortical bone mass in lactating women. Despite the lowered cortical BMD, no changes to femur cross-sectional area were seen and the authors interpreted the mineral losses as having occurred internally through porosity of the endosteum (Laskey et al., 2011). In the present study, ANCOVAs revealed significant increases in humerus medullary area (Es.Ar) with respect to increasing parity (**Table 13**), further highlighting cortical endosteal bone as significantly reduced by increased cycles of pregnancy. Significantly larger medullary

areas have also been observed in rat females who have undergone multiple pregnancies, compared with younger or older nulliparous groups (Miller and Bowman, 2004)

Conclusion

In this study, female reproductive history is significantly associated with several changes to microstructural and macrostructure at the humerus and femur. Overall, parity and IBIavg are associated with increases in osteon sizes at both the humerus (On.Ar) and femur (DOn.Ar). Elevated estrogen levels during pregnancy and the post-weaning period are associated with increases in osteoblastic activity and may help to cumulatively increase overall osteon size. At the midshaft humerus, significant differences in %Po.Ar were seen between low and high %LifeLact groups. Short-term cortical porosity has also been observed experimentally in lactating mice (Liu et al., 2012), with the post-weaning period showing only partial reversal of porosity. The increased porosity seen here with high %LifeLact may be the cumulative result of several long periods of resorption, with only partial recovery each time after weaning. Here, IBIavg is associated with a small but significant decrease in %Po.Ar at the midshaft humerus, suggesting that although the overall effect of high %LifeLact is to increase %Po.Ar in baboons, some additional recovery of porosity is associated with longer IBIavgs. Reproductive history is also associated with significant changes to cross-sectional area, with larger Tt.Ar and Ct.Ar observed in females with high %LifeLact and parity, indicating significant periosteal apposition in these groups. The increased biomechanical strain associated with carrying suckling infants, in addition to the increased maternal body weight during pregnancy, may have stimulatory anabolic effects on the periosteum of cortical bone and may account for the increased total and cortical area measurements seen in these groups. Functionally, increases in periosteal apposition and overall cross-sectional area may help to protect the maternal skeleton from fracture risk despite increases in intracortical porosity (Miller and Bowman, 2004).
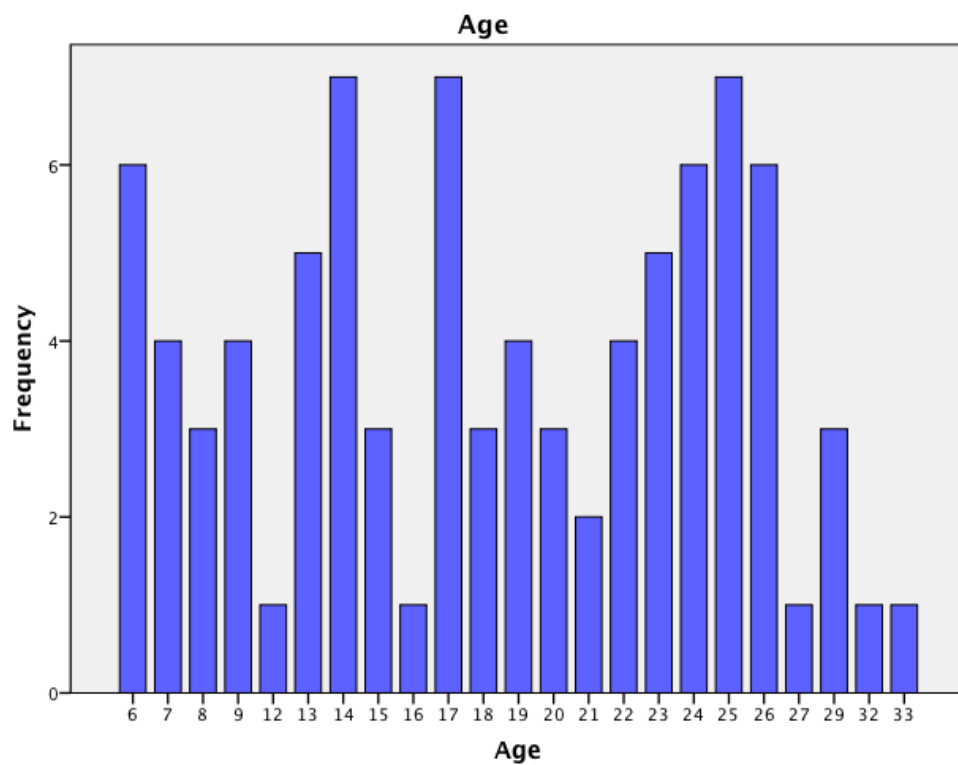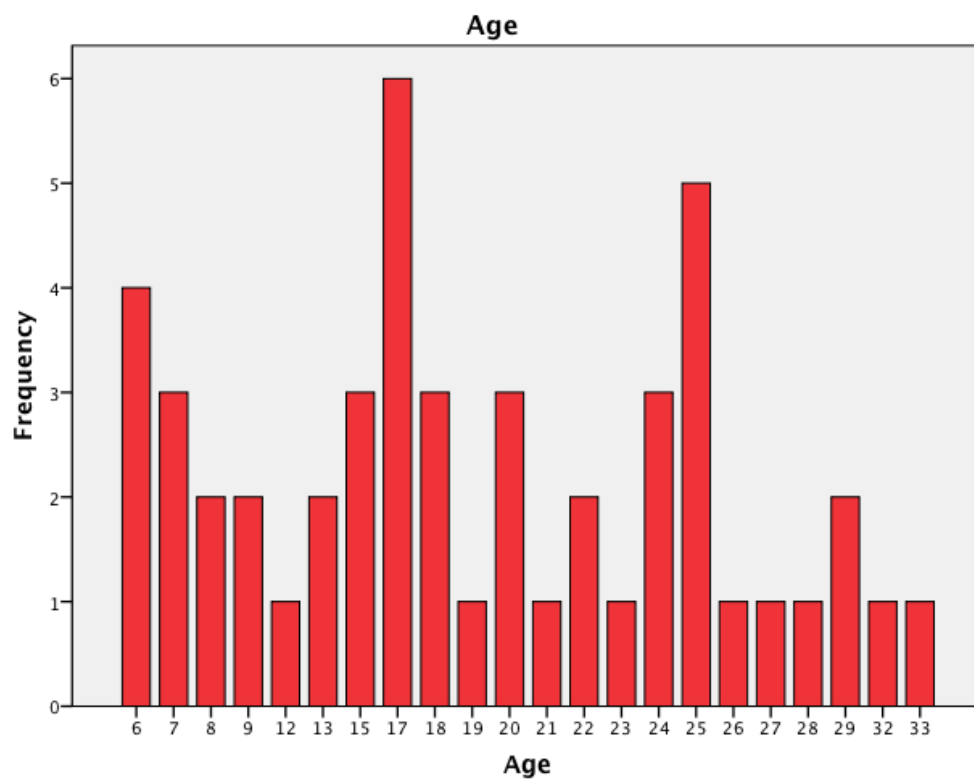
Fig 1: Frequency of female ages (Humerus)

Fig 2: Frequency of female ages (Femur)

Table 1: Bivariate correlations of age and reproductive history variables

	Parity	%LifeLact	IBIavg
Age	.555**	.281**	.455**
Parity		.747**	.069
%LifeLact			.236

Results show Pearson's Correlations values
* = Statistically significant at or below *p* value of 0.05
** = Statistically significant at or below *p* value of 0.01

Table 2: Reproductive profiles by age cohort (Humerus)

Variable	Young Adult (6-18 yrs) N=44		Old Adult (19-33 yrs) N=42		
	Mean	SD	Mean	SD	P
Age	12.11	4.200	24.07	3.262	.000
Weight (kg)	16.80	4.58	17.15	4.21	.713
%LifetimeLact	4.91	8.31	8.62	8.64	.046
IBIavg (days)	314.59	129.73	475.22	129.86	.000
Parity	2.75	3.059	6.07	3.453	.000

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 3: Reproductive profiles by age cohort (Femur)

Variable	Young Adult (6-18 yrs) N=25		Old Adult (19-33 yrs) N=24		
	Mean	SD	Mean	SD	P
Age (years)	12.00	4.655	24.42	3.911	.000
Weight (kg)	17.87	4.62	16.13	2.87	.123
%LifetimeLact	4.05	4.05	7.34	7.34	.143
IBIavg (days)	274.38	138.85	469.22	129.33	.001
Parity	2.44	2.44	5.25	5.25	.005

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 4: Descriptive statistics for female intracortical remodeling dynamics by site

Variable	Humerus (n=87)		Femora (n=49)		
	Mean	SD	Mean	SD	P
Age (years)	18.06	7.0	18.19	7.62	.989
Weight (kg)	17.30	5.26	17.02	3.92	.863
On.Ar (µm²)	5596.34	1685.72	9045.78	2635.13	.000*
H.Ar (µm²)	586.32	90.28	677.30	73.59	.000*
DOn.Ar (µm²)	9443.92	1558.27	8670.28	2334.79	.041*
Dh.Ar (µm²)	806.87	121.39	667.19	93.15	.000*
OPD (#/mm²)	9.86	2.28	8.86	1.77	.005*
%Po.Ar	27.91	9.25	22.80	8.41	.002*
%On.B	7.85	2.11	5.60	1.79	.000*
%C/T	69.20	5.55	69.62	5.44	.668
% C_A/T	49.92	7.96	53.79	7.45	.006*

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 5: Descriptive statistics for female cross-sectional area measurements by site

Variable	Humerus (n=87)		Femur (n=49)		
	Mean	S.D	Mean	S.D.	P
Tt.Ar (mm²)	10.32	1.40	11.97	1.21	.000*
Ct.Ar (mm²)	7.10	.947	8.30	.742	.000*
Es.Ar (mm²)	3.21	.848	3.67	.894	.003*

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 6: Stepwise multiple regression results for intracortical remodeling dynamics (Humerus)

	OPD				%Po.Ar			
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
All females (6-33 yrs) n=87								
Weight	.145(.064)	.296	.027	.071				
IBIavg					-.018(.008)	-.283	.035	.080

Reported covariates are statistically significant at or below *p* value of 0.05
Weight has a small but significant effect on **OPD** (r^2=.071) in the **all adults group**.
IBIavg has a small but significant negative effect on %Po.Ar (r^2=.080) when **all adults** are assessed.

Table 7: Stepwise multiple regression results for intracortical remodeling dynamics (Humerus)

	%C/T				%C$_A$/T			
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
All females (6-33 yrs) n=87								
Parity	-.808(.232)	-.428	.001	.183				
Old adult (19-33 yrs) n=43								
Parity	-.770(.274)	-.440	.008	.193				
%LifeLact					-.367(.137)	-.423	.011	.179

Reported covariates are statistically significant at or below *p* value of 0.05
Results show significant effects of **Parity** on **%C/T** (r^2=.183) in the **all adults** group and **old age group** (r^2=.193). **%LifeLact** has a significant effect on **%C$_A$/T** (r^2=.179) in the **old age group** only.

Table 8: Stepwise multiple regression results for cross-sectional area measurements (Humerus)

	Tt.Ar				Ct.Ar				Es.Ar			
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
All females **(6-33 yrs)** n=87												
%LifeLact	.048 (.017)	.365	.006	.117								
Weight					.055 (.016)	.425	.001	.181				
Weight, %LifeLact					.059 (.015)	.451	.000	.289				
Parity									.128 (.037)	.430	.001	.185
Old adult **(19-33 yrs)** n=43												
%LifeLact	.071 (.019)	.538	.001	.290								
%LifeLact, Weight	.072 (.018)	.552	.000	.420								
Weight					.058 (.021)	.434	.009	.188				
Weight, %Lifelact					.060 (.018)	.451	.000	.381				

Reported covariates are statistically significant at or below *p* value of 0.05

Results show significant effects of **%LifeLact** on **Tt.Ar** (r^2=.117) in the **all adult** age group and **old adult group** (r^2=.290), as well as a combined effect of **%LifeLact and weight** together in the **old age group** (r^2=.420). **Weight and %LifeLact** also significantly affects **Ct.Ar** (r^2=.289) in the **all adult group** as well as in the **old adult group** (r^2=.381). **Parity** significantly affects **Es.Ar** in the **all adult group** (r^2=.185).

Table 9: Stepwise multiple regression results for intracortical remodeling dynamics (Femur)

	On.Ar				Dh.Ar			
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
All females (**6-33 yrs**) n=49								
Age					-8.316(2.330)	-.599	.001	.309
Age, Weight					-10.412(2.387)	-.700	.001	.412
IBIavg	5.554(2.438)	.395	.031	.156				

Reported covariates are statistically significant at or below *p* value of 0.05
Results show significant effects of **IBIavg** on **On.Ar** (r^2=.156) when **all ages** were considered together. **Dh.Ar** is significantly reduced with **age** (r^2=.309) in isolation as well as in **combination with weight** (r^2=.412) when **all adults** were considered together

Table 10: Stepwise multiple regression results for intracortical remodeling dynamics (Femur)

	%C/T				%C$_A$/T			
	Coefficient (SE)	B	P	R^2	Coefficient (SE)	B	P	R^2
All females (**6-33 yrs**) n=49								
Age	-.383(.140)	-.459	.011	.182	-.532(.229)	-.399	.029	.159
Young adult (**6-18**) n=25								

Reported covariates are statistically significant at or below *p* value of 0.05
Results show significant effects of **age** on **%C/T** (r^2=.182) and **%C$_A$/T** (r^2=.159) in the **all adult** group.

Table 11: Stepwise multiple regression results for cross-sectional area measurements (Femur)

	Tt.Ar				Ct.Ar				Es.Ar			
	Coefficient (SE)	B	P	R²	Coefficient (SE)	B	P	R²	Coefficient (SE)	B	P	R²
All females (6-33 yrs) n=49												
Weight	.140(.054)	.439	.015	.192	.100(.028)	.562	.001	.316				
Weight, %LifeLact	.145(.050)	.454	.007	.342								
Young adult (6-18) n=25												
Weight						.710	.010	.504				
%LifeLact									.064(.028)	.580	.048	.336
Old adult (19-33) n=24												
Weight									.131(.055)	.515	.029	.265

Reported covariates are statistically significant at or below *p* value of 0.05
Results show significant effects of **weight** (r^2=.192) and **weight and %LifeLact** (r^2=.342) on Tt.Ar when **all ages** are considered together. **Ct.Ar** is significantly affected by **weight** (r^2=.316) when **all ages** are considered together, as well as in the **young adult** group only (r^2=.504). **Es.Ar** is significantly affected by **%LifeLact** (r^2=.336) in the **young adult** group and weight in the **old adult** group (r^2=.265).

Table 12: ANCOVA for Nulliparous **(Group 0)**, Low parity **(Group 1)**, and High parity **(Group 2)***Age (Humerus)

Repro. Group	On.Ar		H.Ar		DOn.Ar		Dh.Ar	
	μ	S.E	μ	S.E	μ	S.E	μ	S.E
Group 0 n=20	5030	298.7	563.9	21.80	9422.77	437.3	831.2	26.39
Group 1 n=18	5118	371.7	587.8	20.32	8934.53	407.7	791.7	24.51
Group 2 n=15	5882	454.7	599.3	24.86	10376.9	498.7	816.9	30.10
P-value	.353		.604		.082		.535	
Post-hoc	N.S.		N.S.		1v2		N.S.	

Repro. Group	OPD		%On.B		%Po.Ar		%C/T		% C$_A$/T	
	μ	S.E	μ	S.E	μ	SE	μ	SE	μ	SE
Group 0 n=20	10.00	.61	7.86	.62	25.12	2.37	69.16	1.24	51.75	1.81
Group 1 n=18	10.51	.57	8.30	.58	27.32	2.21	71.07	1.16	51.64	1.69
Group 2 n=15	9.484	.69	8.56	.71	30.41	2.70	66.48	1.42	46.32	2.07
P-value	.496		.791		.413		.043		.108	
Post-hoc	N.S.		N.S.		N.S		1 v 2		1 v 2	

Nulliparous= 0 birthed offspring; Low parity= 1-3 birthed offspring; High parity= 9+ birthed offspring
Tukey post-hoc indicates difference between groups, *p* value at or below 0.05

Table 13: ANCOVA for Nulliparous **(Group 0),** Low parity **(Group 1),** and High parity groups **(Group 2)*** Age   (Humerus)

Repro. Group	Tt.Ar		Ct.Ar		Es.Ar	
	Mean	S.D.	Mean	S.D	Mean	S.D.
Group 0 n=20	9.816	.279	6.773	.168	3.043	.188
Group 1 n=18	9.848	.260	6.972	.157	2.876	.175
Group 2 n=15	10.767	.319	7.095	.192	3.673	.214
P-value	.061		.510		.018	
Post-hoc	1 v 2 0 v 2		N.S.		1 v 2 0 v 2	

Nulliparous= 0 birthed offspring; Low parity= 1-3 birthed offspring; High parity= 9+ birthed offspring

Tukey post-hoc indicates difference between groups, *p* value at or below 0.05.

Table 14: ANCOVA for 0%LifeLactation (**Group 0**), low %LifeLactation (**Group 1**), and high %LifeLactation groups (**Group 2**)* Age (Humerus)

Repro Group	On.Ar		H.Ar		DOn.Ar		Dh.Ar		OPD	
	μ	S.E	μ	S.E	μ	S.E	μ	S.E	μ	S.E
Group 0 n=37	5339	296	593	14	9144	294	805	22.6	10.2	.42
Group 1 n=11	5691	533	557.1	25.23	9662	529	797	40.6	8.48	.76
Group 2 n=17	5620	427.4	595.1	20.25	10091.9	424.5	844.57	32.57	9.96	.61
P-value	.815		.885		.222		.538		.139	
Post-hoc	N.S.		N.S		N.S.		N.S.		N.S.	

Repro Group	%On.B		%Po.Ar		%C/T		% C$_A$/T	
	μ	S.E	μ	SE	μ	SE	μ	SE
Group 0 n=37	7.94	.41	27	1.67	70	.846	51.1	1.26
Group 1 n=11	6.89	.73	23.16	3.00	69.95	1.52	53.53	2.27
Group 2 n=17	8.60	.59	33.02	2.42	70.01	1.22	46.91	1.82
P-value	.175		.024		.995		.094	
Post-hoc	N.S.		1vs 2				1 v 2	

0= 0%LifeLactation; **1**= 1-4% LifeLactation; **2**= 15+% LifeLactation
Tukey post-hoc indicates difference between groups, *p* value at or below 0.05

Table 15: ANCOVA for 0%LifeLactation **(Group 0),** low %LifeLactation **(Group 1),** and high %LifeLactation groups **(Group 2)*** Age   (Humerus)

	Tt.Ar		**Ct.Ar**		**Es.Ar**	
Repro. Group	Mean	S.D.	Mean	S.D	Mean	S.D.
Group 0 **n=37**	9.869	.180	6.875	.103	2.994	.127
Group 1 **n=11**	10.927	.323	6.960	.186	3.067	.229
Group 2 **n=17**	10.618	.259	7.387	.149	3.231	.183
P-value	.074		.025		.595	
Post-hoc	0 v 2		O v 2		N.S.	

0= 0%LifeLactation; **1**= 1-4% LifeLactation; **2**= 15+% LifeLactation
Tukey post-hoc indicates difference between groups, *p* value at or below 0.05
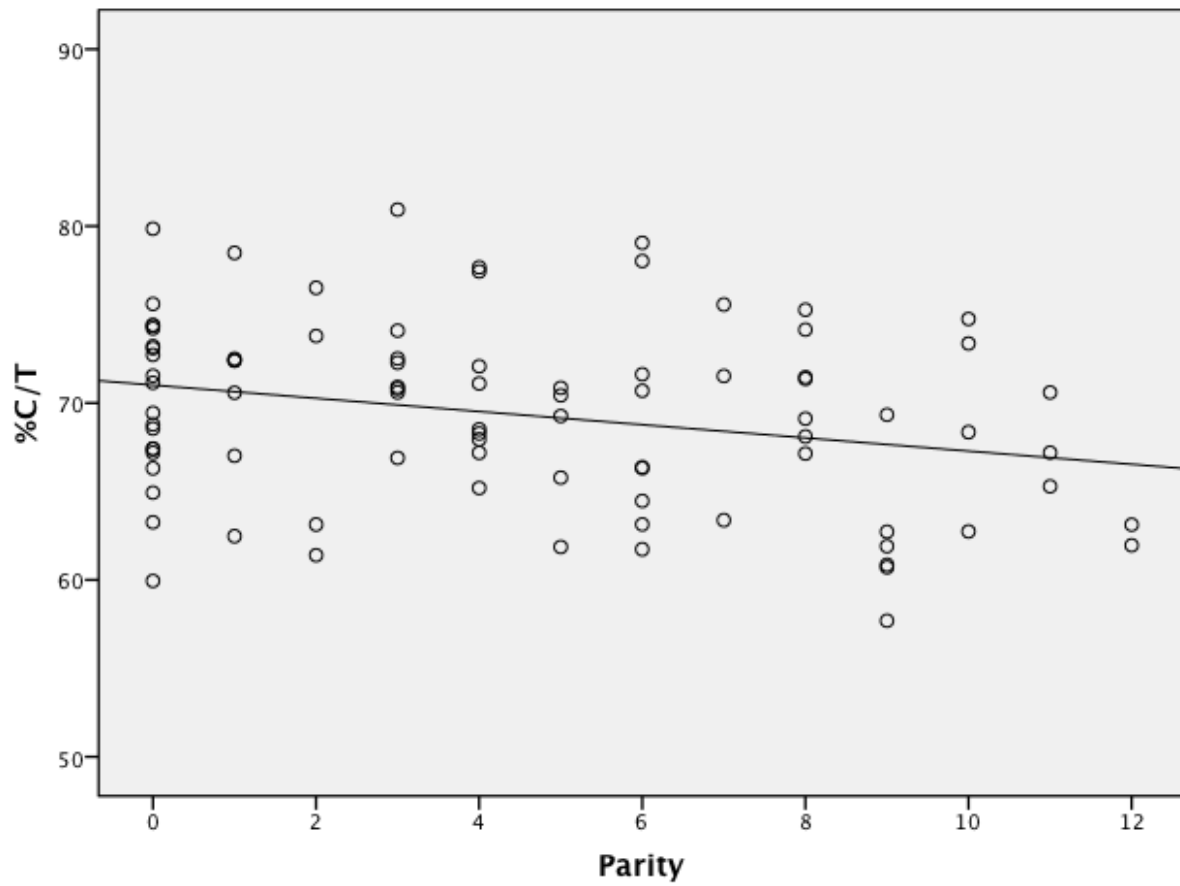
Fig. 3: Scatterplot showing negative relationship of %C/T and parity in the humerus
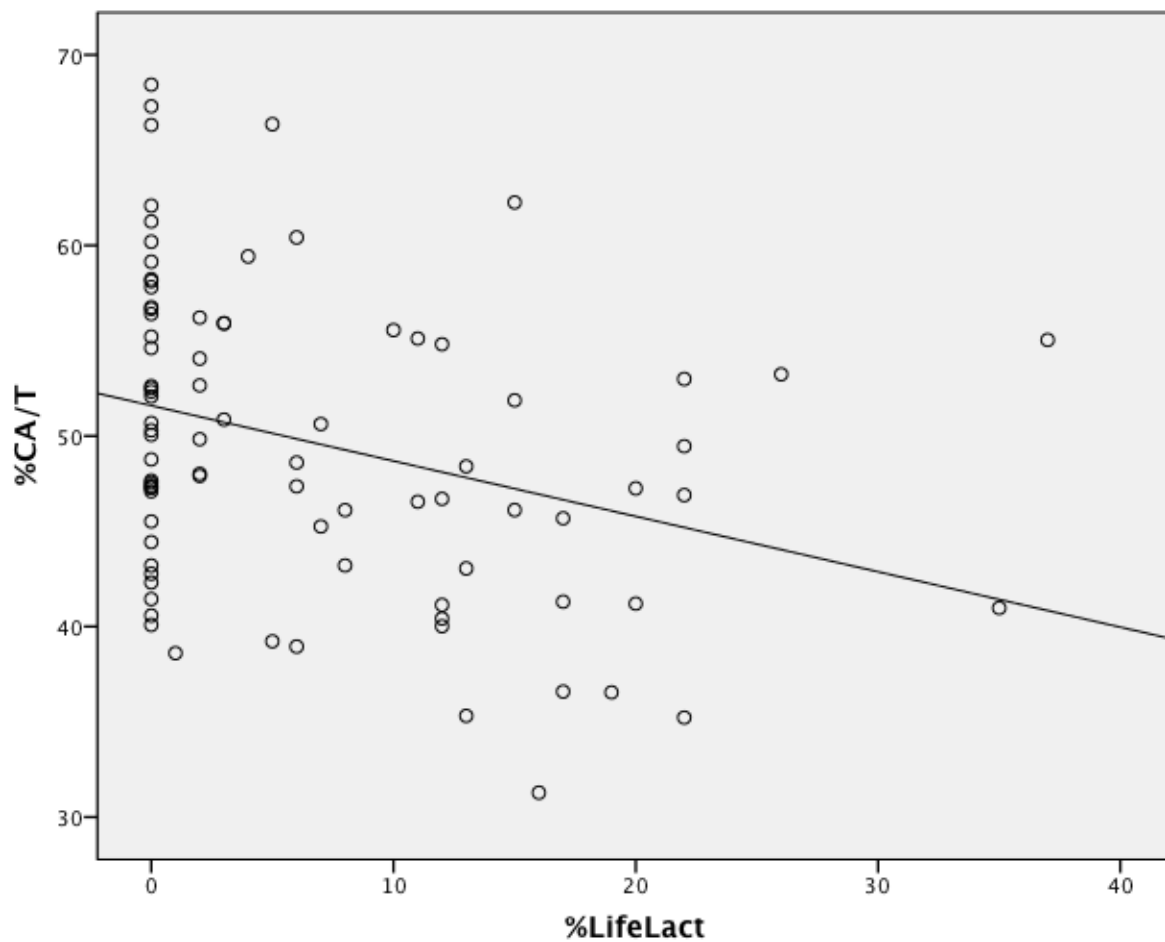
Fig 4: Scatterplot showing negative relationship of %LifeLact with %C_A/T (Humerus)

Chapter 6

Effect of reproduction on lumbar trabecular microarchitecture

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Introduction

Trabecular bone is a continuous 3-D network of bars and plates, its microstructure composed primarily of vertical columns of bone connected by horizontal struts (Mosekilde, 1993; Parfitt, 1992). Trabecular bone is highly heterogeneous; in addition to varying densities it possesses varying orientations and degrees of orientation. Characterization of trabecular architecture is typically assessed through means of the number of trabeculae in a given volume (Tb.N), their average thickness (Tb.Th), the average distance between adjacent trabeculae (Tb.Sp), and the degree to which trabeculae are connected to each other (Conn.D). Previously, assessment of trabecular architecture was possible only by 2-D histomorphometry, but recent advances in high-res microcomputed tomography and magnetic resonance imaging now allow for 3D assessments of biopsied trabecular structures (Hildebrand et al., 1999; Majumdar et al., 1998; Genant et al., 2000). Although bone density is among the strongest predictors of mechanical behavior of trabecular bone, theoretical and empirical analyses have shown that aspects of trabecular microarchitecture influence bone strength as well (Rice et al., 1988; Keaveny et al., 2001; Currey, 1986). Trabecular microstructural variability (e.g. bone quality) has several significant effects on mechanical properties and tissue stress in humans (Yeni et al., 2008) and is important to measure alongside bone volume (e.g. bone quantity). In general, trabecular microarchitecture is strongly correlated with bone volume (Hildebrand et al., 1999; Goulet et al., 1994; Bouxsein et al., 1997), and connectivity of the trabecular network is especially critical to bone strength (Silva et al., 1997).

The vertebral body site has traditionally been preferred for sampling as a proxy for whole-body cancellous bone health, in particular lumbar sites rather than thoracic or caudal spinal segments, because the volume of the lumbar vertebral bodies is the greatest and offers the most micro-architectural network to sample (Bouxsein et al., 2010). Human vertebral cancellous bone is also one of the primary sites affected by short-term skeletal resorption and mobilization of calcium during the periods of pregnancy and lactation (e.g. Olausson et al., 2008; More et al., 2001; Laskey and Prentice, 1999). A meta-data analysis (Olausson et al., 2012) found mean lumbar BMD changes ranged from -4.5 to -0.9% during pregnancy and -7.5 to -2.8% during lactation. In general, studies have shown that greater losses occur in trabecular over cortical bone and from axial than appendicular sites (Laskey et al., 2011; Glerean et al., 2010; Hellmeyer et al., 2006; Chan et al., 2005; Pluskiewicz and Drozdzowska, 2002; Matsushita et al., 2002; Honda et al., 1998; Kurl et al., 2002). However, the long-term effects of parity and lactation with respect to long-term trabecular bone health are not uniformly established, with some studies showing positive (e.g. Hiller et al., 2003; Cure-Cure et al., 2002) and others showing negative effects (e.g. Canal-Macias et al., 2012; Schnatz et al., 2010). Retrospective studies in old world monkeys have also been conflicting, with one showing a positive effect of parity on vertebral BMD in macaques (Cerroni et al., 2003) and another showing a negative effect of parity on vertebral BMD in baboons (Havill et al., 2008). However bone mass measurements provide only

a partial assessment of vertebral trabecular health, and the lack of understanding about the microarchitectural that accounts for low or high BMD leads to conflicting results. Characterization of the trabecular microarchitecture that accompanies increasing parity and lactation durations could lend considerable clarity into how reproductive histories affect vertebral bone health over the aging process.

Study Goals

The goal of this retrospective study is to assess the effects of reproductive history on lumbar trabecular architecture and quantity in baboons. The hypothesis tested in this study is that reproductive histories will be informative in explaining bone volume and trabecular micro-architectural variation throughout the baboon lifespan. A previous retrospective analysis of reproductive history and lumbar BMD in baboons found a negative effect of parity (Havill et al., 2008); therefore, I further hypothesize that the overall effect of high parity and extended lactation will result in the deterioration of the microstructural network and connectivity.

Methods

L1 sample and micro-CT analysis:

During the period of 2004-2008 at the SNPRC, frozen vertebral spines (the segment of T12-L7) were taken at animal necropsy, wrapped in saline-soaked gauze, placed in air-tight plastic bags and kept frozen until sample preparation. During the summer of 2012, I traveled to the SNPRC and located the frozen vertebral columns for each baboon chosen in the dissertation sample population. A small percentage of the animals did not have vertebrae taken at necropsy or had damaged lumbar vertebrae. From the remaining samples, the L1 vertebra was selected for inclusion in this thesis. The cylindrical core sampling methodology was selected for the assessment of trabecular bone micro-architecture. Cylindrical cores are preferred because they are a precise and standardized means of bone sampling and cores can subsequently be used in mechanical strength testing for further analysis (e.g. Yeni et al., 2011; Yeni et al., 2008; Hou et al., 1998). Lumbar height and length was measured for all female L1 samples and a core size of 8mm x 12mm was selected as an appropriately scaled region of interest. Diamond-coated coring drill bits were ordered from Starlite Tools (800-727-1022). All coring work was performed in the Orthopaedic Biomechanics Laboratory (PI: Dr. Tony Keaveny) at U.C. Berkeley. Frozen L1 samples were first oriented superiorly (i.e. with the articulation for T12 facing upward) with lamina anteriorly, and midpoint was calculated using the total length across the superior surface of the vertebra (from left to right) as well as the total width (anterior to posterior). From this midpoint, the center of the core was fixed 5mm laterally to the right in order to avoid the vertebral artery that supplies blood to the interior of the vertebral body (**Fig. 8**). Coring was performed continuously under water to reduce friction.

A total of n=24 vertebral L1 cores from females with variable ages, parities and %LifeLact were analyzed at the J.D. Wheat Veterinary Orthopedic Research Laboratory, School of Veterinary Medicine on the U.C. Davis campus. Due to the high sample costs and lengthy reconstruction times of micro-CT analysis, a female-only pilot sample of n=24 was deemed appropriate for the budgetary and time constraints of this doctoral thesis. A cylinder of

approximately 8 mm in diameter and 12 mm in length was centered in a scaffold core and imaged (70 kVp, 114 μA, 300 ms integration time, average of 3 images) using a high- resolution μCT specimen scanner (μCT 35, Scanco Medical; Bassersdorf, Switzerland). 820 contiguous slices of 2,048 x 2,048 pixels were imaged with 10 μm resolution and slice thickness (voxels). On each of the 2-dimensional images a region of interest was manually drawn. The region of interest excluded the external rim of the core to avoid inclusion of broken and partial trabeculae pieces resulting from the coring process. Values from all continuous slices were averaged for each core and this average was used in all subsequent calculations and analyses.

Standard measures of trabecular architecture are assessed, including bone volume, trabecular thickness, trabecular separation, trabecular number, connective density and the structural model index (Parfitt et al., 1987). These measures reflect both quantitative and qualitative aspects of trabecular bone.

1. **BV/TV**, bone volume fraction **(%)**: defined as cancellous bone volume, or the percent of total marrow cavity that is occupied by cancellous bone (both mineralized and non-mineralized). Low= deficit in bone mass

2. **Tb.Th**, trabecular thickness **(μm)**: defined as the mean distance across individual trabeculae

3. **Tb.N**, trabecular number **(1/mm):** defined as the average number of trabeculae per unit length

4. **Tb.Sp,** trabecular separation **(μm):** defined as the mean distance between trabeculae

5. **Conn.D**, connectivity density **(1/mm^3):** defined as the degree (or redundancy) of trabecular connectivity

6. **SMI**, structure model index: defined as the characterization of the structure of trabeculae as either rod-like (vertical) or plate-like (horizontal). SMI will be 0 for parallel plates and 3 for cylindrical rods (Hildebrand and Ruegsegger, 1997a)

Statistics:

Normality tests (the Shapiro-Wilk test as well as skewness and kurtosis) were first assessed to determine the appropriateness of parametric analyses. Several variables did not pass normality tests (BV/TV, SMI, Tb.Th) and nonparametric tests were used subsequently to analyze them. Scatterplots helped identify statistical outliers and general trends in trabecular architecture parameters with age, sex, and reproductive variables. Several females were removed from regressions and t-tests for being two or more standard deviations away from the norm in multiple variables, reducing the overall number of samples to 19 in those analyses. Descriptive statistics and t-tests (Mann-Whitney tests for non-normal variables) were performed to show significant differences in trabecular variables between young and old age cohorts. Linear regressions were

carried out to assess if age and reproductive history was significant in explaining variation in trabecular architecture. Cross-method bivariate correlations (using Pearson's tests for normal variables and Spearman's tests for non-normal variables) were carried out to assess the relatedness of cortical and trabecular quantity and quality measurements.

Results

Age:

        Table 1 shows the reproductive profiles for the young adult and old adult cohorts. T-tests show that that the young adult group and old adult group differ significantly in parity and IBIavg. **Table 2** demonstrates the trabecular variable averages and standard errors for each age cohort, with p-values to show significant differences between the groups. No significance was seen between young and old age cohorts in any parameters (**Table 2**). Bivariate correlations (using both Pearson's and Spearman's tests) showed that age is only significantly correlated with Tb.Th in this sample (**Table 3**). However, when plotted, the data reveals some additional interesting trends. Overall, BV/TV appears to increase with age until late in the second decade of life, where it rapidly declines (**Fig 1**). This is the period associated with natural menopause and decreased estrogen levels in captive baboons (Martin et al., 2003). Rapid declines in lumbar trabecular bone at menopause have also been seen in humans (e.g. Riggs et al., 2008) and baboons (Havill et al., 2008). The reduction in BV/TV observed here appears to primarily correspond with a decline in Tb.Th (**Fig. 2**), which is significantly correlated with age here (**Table 3**).

Reproductive history:

        After the removal of significant outliers, regression analyses and t-tests (Mann-Whitney tests for nonparametric variables) did not find any significant effects of parity, %LifeLact, or IBIavg on trabecular architecture in this sample. However, detailed analysis was performed to assess if the statistical outliers seen in the age plots (i.e. **Fig.s 1, 2**) had informative aspects of reproductive history.

        Two females had recently given birth before their deaths, and showed significantly different means in several variables. One female (6746) died less than one month after parturition and showed very high Tb.N, very high BV/TV (**Fig. 1**), high Conn.D, and low Tb.Sp and SMI. Another female (10114) died four days after parturition and showed many of the same changes, including high Tb.N, high Conn.D, and low Tb.Sp. Both of their infants died soon after birth and these females did not lactate. Parity differences may explain the variables that are not similar between these females (BV/TV, SMI) and are further explored below. Differences in bone quality that are shared between these females and may be unique to pregnancy and parturition-related short-term alterations in trabecular bone are increased Tb.N (**Fig. 3**), low Tb.Sp, and and high ConnD (**Fig. 4**). These are further discussed with respect to the literature.

        Parity, %LifeLact and IBIavg were assessed with respect to the rest of the statistical outliers observed in the age plots. A plot of BV/TV and age showed female 1x3469 to have very

high BV/TV (**Fig. 1**). A plot of BV/TV with parity (**Fig. 5**) shows female 1x3469 to have unusually high BV/TV for her parity (8). However, females with similar parities (9 and 10) had significantly lower BV/TV. Her %LifeLact (11%) and IBIavg (368 days) were similar to age-matched high parity females exhibiting low BV/TV. Unfortunately reproductive history does not explain this unusual result.

However, there is an additional interesting outlier with respect to %LifeLact. The female (1C1437) with the highest %LifeLact (34%) is observed to have the highest Tb.Sp mean in the sample (**Fig. 2**), even compared to other females with high %LifeLact (18-20%). It is possible that the significant mineral resorption associated with extended lactation could cumulatively result in larger spaces between trabeculae. Interestingly, this outlier female has abnormally low Tb.N and ConnD (an outlier in that respect as well), so the large Tb.Sp seen is a result of significantly low Tb.N and connectivity. Her Tb.Th is the normal range, so the issue is not significantly thinned trabeculae, but lower total number of trabeculae and larger spaces between them. Confounding effects of aging are not an issue in this case, as she is only 19 years old and the older individuals in the sample (as old as 33 years) have very different results in Tb.Sp, Tb.N and ConnD. Though interesting, this is a single example and further analysis testing Tb.Sp, Tb.N, and ConnD with extensive lactational history is needed. Future analyses investigating the cumulative effects of extensive lactation should focus on a larger sample of females with 30%+ LifeLact in order to further assess the possibility of this aspect of reproductive history in significantly increasing trabecular spacing and number.

Correlations with cortical quantity and quality:

Bivariate correlations show some significant relationships of trabecular architecture with intracortical remodeling (**Table 4**), but these are not meaningful in explaining coupled bone loss at both cortical and trabecular sites. The literature in humans currently indicates that cortical and trabecular bone does not follow the same trajectory of deterioration with aging. In general, the preservation of cortical bone is seen until midlife in women (and until even later in men), whereas trabecular bone quantity begins to deteriorate as early as the third decade of life (Riggs et al., 2008).

Discussion

Human vertebral cancellous bone is typically one of the primary sites for age-related bone loss, showing reduced cortical thickness and density, decreased plate number, and decreased trabecular connectivity (Walker et al., 2013) with increasing age. The literature shows an average vertebral BMD decline of -18% per decade in women (reviewed in Silva and Jepsen, 2013). Specifically in the lumbar spine, BMD loss begins in the third decade of life, and accelerates at peri-menopause; this is in opposition to appendicular trabecular sites where bone loss is more or less constant with age in women (Riggs et al., 2008). Beginning at peri-menopause, spinal trabecular vBMD declines at a rate of -16%/decade and further accelerates to an average of -26% in post-menopausal women (Riggs et al., 2008). In this study, female baboon trabecular BV/TV appears to increase with age until late in the second decade of life

when it declines rapidly (**Fig. 1**). Both captive and wild baboons begin to experience complete cessation of reproductive cycling with decreasing estrogen levels (Honore et al., 2000) on average at 26 years of age (*captive* Martin et al., 2003; *wild* Packer et al., 1998). The rapid decline in lumbar BV/TV seen here at the age of menopause corresponds with the bone mineral losses observed in humans at this period (e.g. Riggs et al., 2008). The loss in BV/TV here appears to be related to loss of trabecular thickness, which is significantly correlated with age (**Table 3**) and which also shows a decline at menopause (**Fig. 2**). Although statistical significance in BV/TV was not found between young and old age cohorts here (**Table 2**), this is unsurprising given that age-related changes are not dramatically apparent until menopause. Little significance of age was also observed in a larger micro-CT study of lumbar cores in baboons (Havill et al., 2010). In baboons, aging does not appear to have strong effects on trabecular bone quantity and quality until very old age, and a much larger sample of elderly individuals (i.e. age 28+ years) is needed to properly assess age effects in lumbar cancellous bone (Havill et al., 2010). A large percentage of vertebral architecture in this species may also be related to genetics. Havill et al., (2010) observed large genetic effects in BV/TV (48%), ultimate stress (51%) and toughness (59%), though Tb.Th, Tb.N, and Tb.Sp in relation to genetics have not yet been assessed directly.

Two females had recently given birth before their deaths, and showed significantly different means in several variables. These females (6746 and 10114) both died within a month of parturition. Their infants, too, died soon after birth and these females did not lactate. Unique differences in bone quality that are shared between these females and may be related to short-term alterations in trabecular bone related to pregnancy and post-parturition are increased Tb.N (**Fig. 3**), low Tb.Sp, and and high Conn.D (**Fig. 4**). In humans, a histomorphometric study of iliac cancellous biopsies taken at the time of scheduled, full-term c-section also observed a significant increase in trabecular number through a process of pregnancy-related trabecular proliferation (Shahtaheri et al., 1999). Although the mechanism that results in these changes is unclear, Shahtaheri et al., (1999) hypothesize that the appearance of new intratrabecular resorption cavities transformed previously thickened trabecular plates into multiple thinner ones (**Fig. 6**), increasing both the Tb.N and Conn.D. These data are compatible with the trabecular quality changes seen in the two baboon females who died shortly after parturition here.

This pilot study also shows a potentially interesting relationship between lactational history and long-term alterations to bone quality. A non-elderly female with a history of very high lactation (%LifeLact=34%) is a statistical outlier in trabecular architecture in several respects. This outlier female (1C1437) has abnormally high Tb.Sp though other females with increased %LifeLact (18-20%) also show increases in Tb.Sp **(Fig. 7).** Her Tb.N and ConnD is abnormally low (she is an outlier in that respect as well), so the large average Tb.Sp observed is a result of significantly low Tb.N and Conn.D. Interestingly, her Tb.Th is the normal range, so the issue is not related to significantly thinned trabeculae, but rather the lower total number of trabeculae and larger spaces between them. Confounding effects of aging are not an issue in this case, as she is only 19 years old and the older individuals in the sample (as old as 33 years) have very different results in Tb.Sp, Tb.N and ConnD. In a micro-CT study of the effects of lactation on cancellous bone in mice, lactation was associated with marked deterioration in trabecular architecture, as compared to nulliparous controls (Liu et al., 2012). Plate number decreased as much as 19-21% during lactation, as well as plate-rod and plate-plate junction densities (P-R and

P-P Junc.D) by 48%-75% and 66%-82% (Liu et al., 2012). After weaning, several differences in trabecular architecture still remained. The P-R ratio was still lower in recovered mice (-35%), as well as plate trabecular number in the tibia and femur. These results suggest that some aspects of trabecular architecture deterioration may not fully recover after lactation. This effect may be exacerbated in individuals who lactate quite extensively (30+ %LifeLact), as seen here.

These data, though preliminary, show a possibly interesting interplay between pregnancy, lactation, and bone quality in baboons. Although short-term pregnancy-related changes may include increased Tb.N and Conn.D, the large resorption of cancellous bone that occurs during multiple cycles of long lactation deteriorates these "new" struts, leaving behind large spaces, a reduced number of trabeculae, and decreased connectivity. However, both these short and long-term alterations are based on a small number (n=3) of outliers and need to be more rigorously tested in a larger sample group.

The long-term effects of extended lactation on bone quantity and quality are currently unclear. In humans, total duration of breastfeeding per child may be a significant factor in osteoporosis and fracture risk. There is some evidence that histories of extended breastfeeding (longer than one year per child) in combination with high parity may be associated with low pre- and post-menopausal BMD, especially in women who experience low calcium intake, low dietary nutrition, and low socioeconomic status (Henderson et al., 2000; Dursun et al., 2006; Demir et al., 2008; Hopkinson et al., 2000; Okyay et al., 2013). A significant negative correlation was seen with (retrospective) average breastfeeding durations of 24-36 months and BMD in Spanish women (Rojano-Mejia et al., 2011), and a similar negative relationship with BMD was observed with histories of breastfeeding past one year in Turkish women (Okyay et al., 2013). Extended periods of lactation may impair the natural process of bone recovery, or further exacerbate the resorptive bone changes seen during typical lactation. More et al. (2001) reported that recovery of lactational bone loss was seen in all participants except in women who breastfed for a longer (>1 year) period of time. In a multivariate analysis of reproductive history and osteoporosis risk, extended breastfeeding was the single greatest predictor of the development of osteoporosis (odds ratio: 12.92; 95% confidence interval, 3.1–52.6) (Okyay et al., 2013).

Conclusion

This retrospective study shows several potential relationships between trabecular bone quality and quantity and reproductive history. Two females who died recently (<1 month) after parturition showed increased trabecular number and connectivity relative to other females without recent births. These same architectural parameters have also been observed in cancellous bone at the end of pregnancy in women, and may be related to a proliferation in the number of struts through the appearance of new intratrabecular resorption cavities (Shahtaheri et al., 1999). The long-term ramifications of these architectural changes are unclear, but here the female with the highest lactational history (34% total %LifeLact) showed significantly increased trabecular spacing, low trabecular number, and low connectivity over other females. Lactation-related reductions in trabecular number and connectivity have also been observed in mice (Liu et al., 2012) and it is possible the increased proliferation of struts that occurs during pregnancy is dramatically resorbed during long periods of lactation, leaving behind a network of fewer struts with low connectivity. The sample size assessed here is not large enough for rigorous testing of this hypothesis, and future work should examine the relationship of extended lactation on bone quality in baboons using a larger sample of females with high %LifeLact.

Table 1: Reproductive profiles by age cohort (L1 vertebral core)

	Young Adult (6-18 yrs) N=12		Old Adult (19-33 yrs) N=7		P
	Mean	SE	Mean	SE	
%LifetimeLact	3.168	1.811	11.08	4.54	.080
IBIavg (days)	132.2	55.08	365.45	69.17	.004
Parity	2.091	.6935	7.000	1.480	.018

Statistical difference between the sexes was tested using a Student's *t*-test
* = Statistically significant at or below *p* value of 0.05.

Table 2: Descriptive statistics for trabecular architecture by age cohort (L1 vertebral core)

Variable	Young adult (6-18 years) N=12		Old adult (19-33 years) N=7		
	Mean	SE	Mean	SE	P
Age (years)	12.34	1.019	25.83	1.75	.000
BV/TV	.2782	.0091	.3011	.0324	.411
ConnD	13.56	1.921	10.81	1.413	.333
SMI	-.2992	.1152	-.6002	.3122	.296
Tb.N	1.772	.0735	1.645	.0745	.273
Tb.Th	.1381	.0034	.1804	.0274	.273
Tb.Sp	.5331	.0213	.5904	.0955	.201

Statistical difference between the sexes was tested using a Student's *t*-test for normal variables (ConnD, Tb.N, Tb.Sp) and Mann-Whitney test for non-normal variables (BV/TV, SMI, Tb.Th)
* = Statistically significant at or below *p* value of 0.05.

Table 3: Bivariate correlations of trabecular architecture (L1 vertebral core)

	BV/TV	ConnD	**TRISMI**	Tb.N	**Tb.Th**	Tb.Sp
Age	.216	.017	-.186	.056	.204*	.027
BV/TV		.098	-.875**	.579**	.472*	-.411
ConnD			.156	.813**	.611**	-.759**
TRISMI				-.319	-.491*	.144
Tb.N					-.305	-.956**
Tb.Th						.405

Unbolded show results of Pearson's Correlations values (ConnD, Tb.N, Tb.Sp)
Bolded show results of Spearman's Correlations values (**BV/TV, SMI, Tb.Th**)
* = Statistically significant at or below *p* value of 0.05
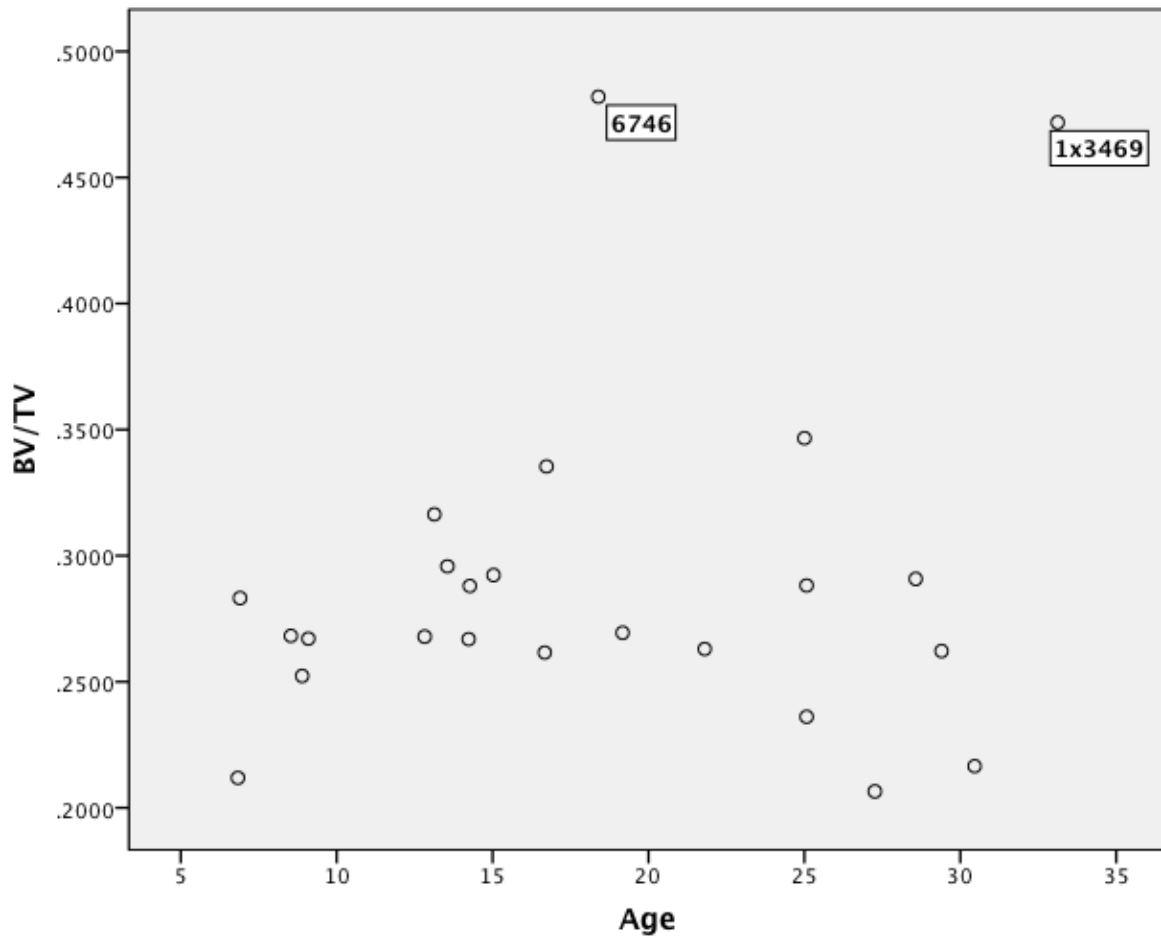** = Statistically significant at or below *p* value of 0.01

Fig. 1: Scatterplot showing age and BV/TV. Though not significant, BV/TV appears to increase with age and rapidly decline near the age of menopause in baboons (26 years). Two statistical outliers are observed and are discussed with respect to their reproductive histories.
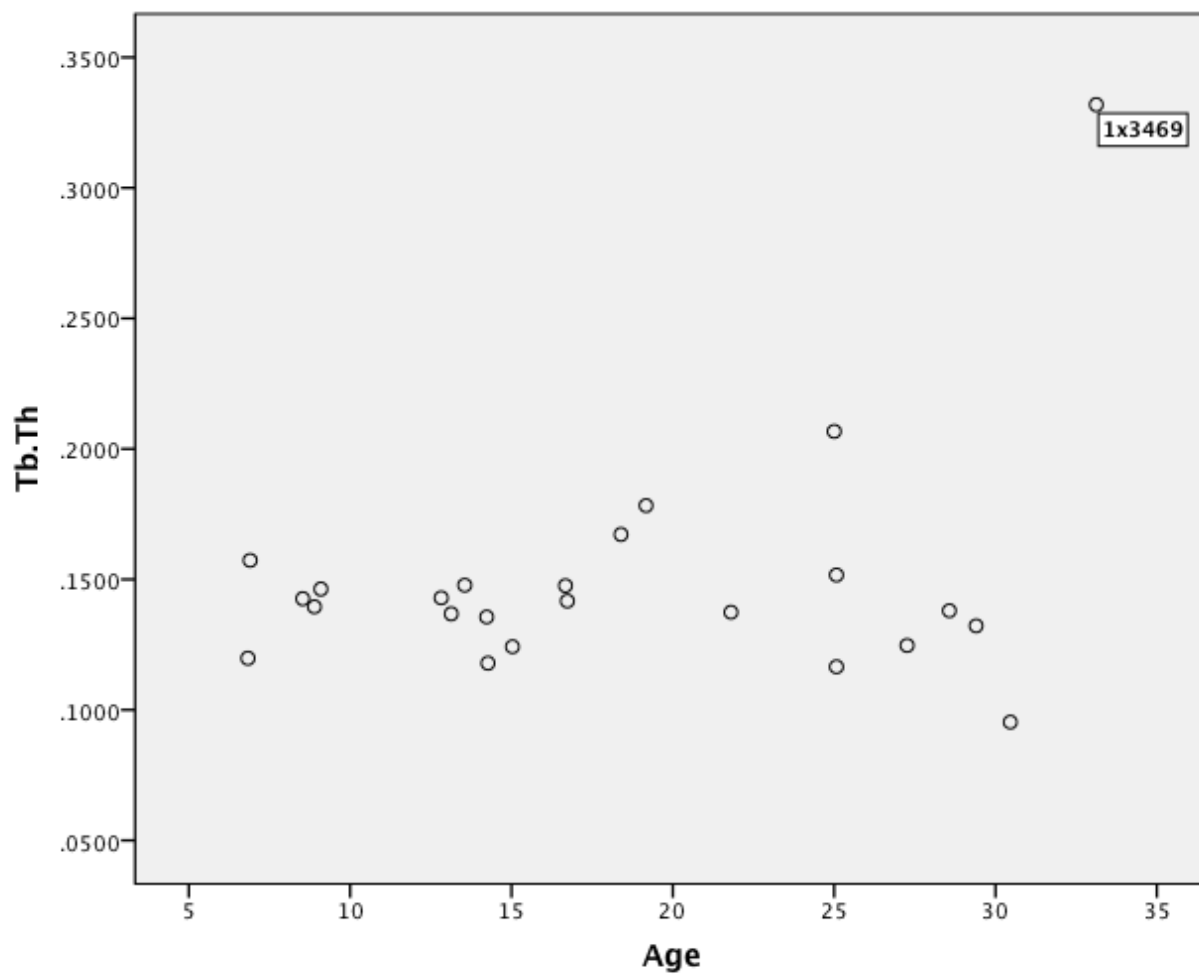
Fig. 2: Scatterplot showing age and Tb.Th. Tb.Th also shows a decline with age, around the time of menopause in baboons (age 26)
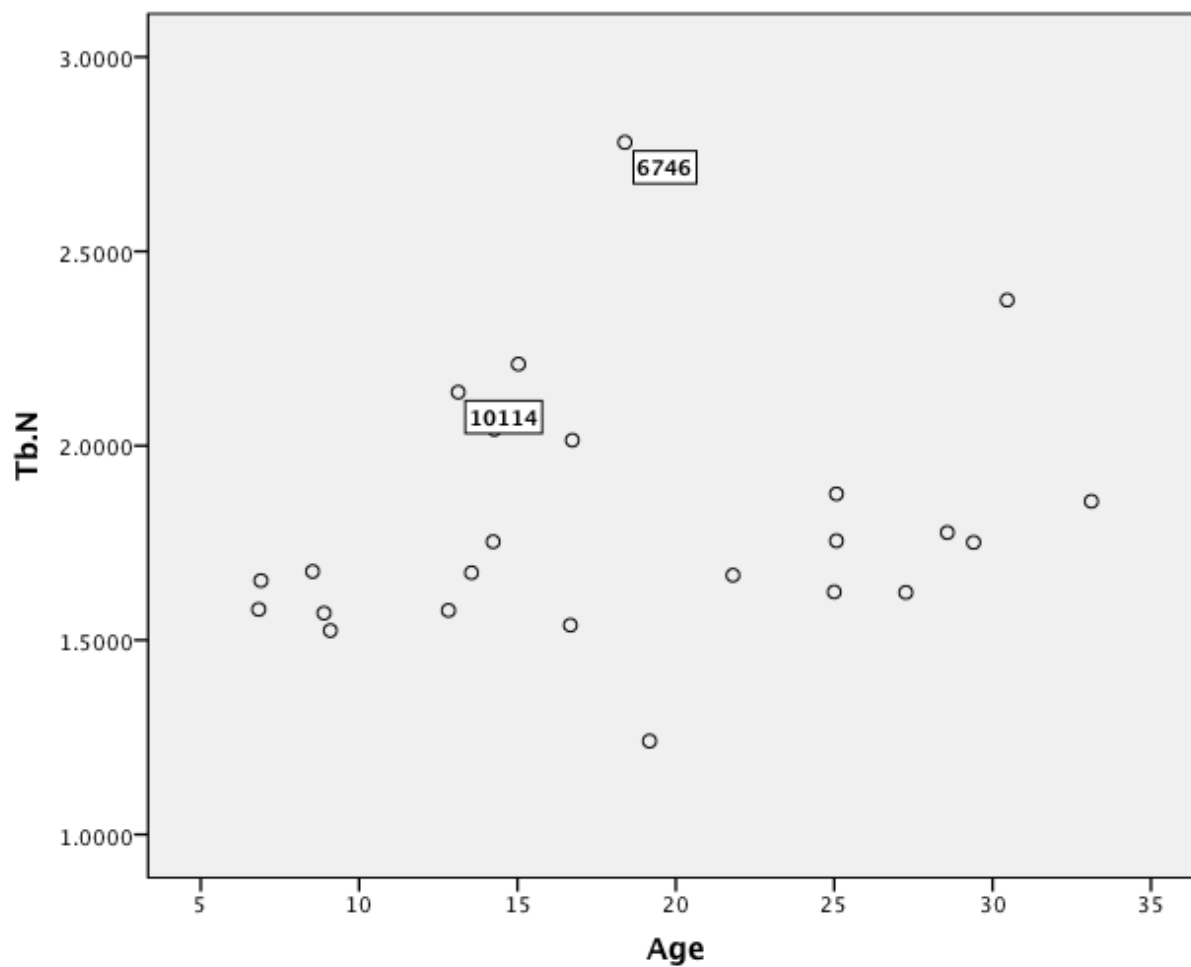
Fig. 3: Scatterplot showing age and Tb.N. Two females (6746, 10114) recently gave birth before their deaths and show unusually high Tb.N.
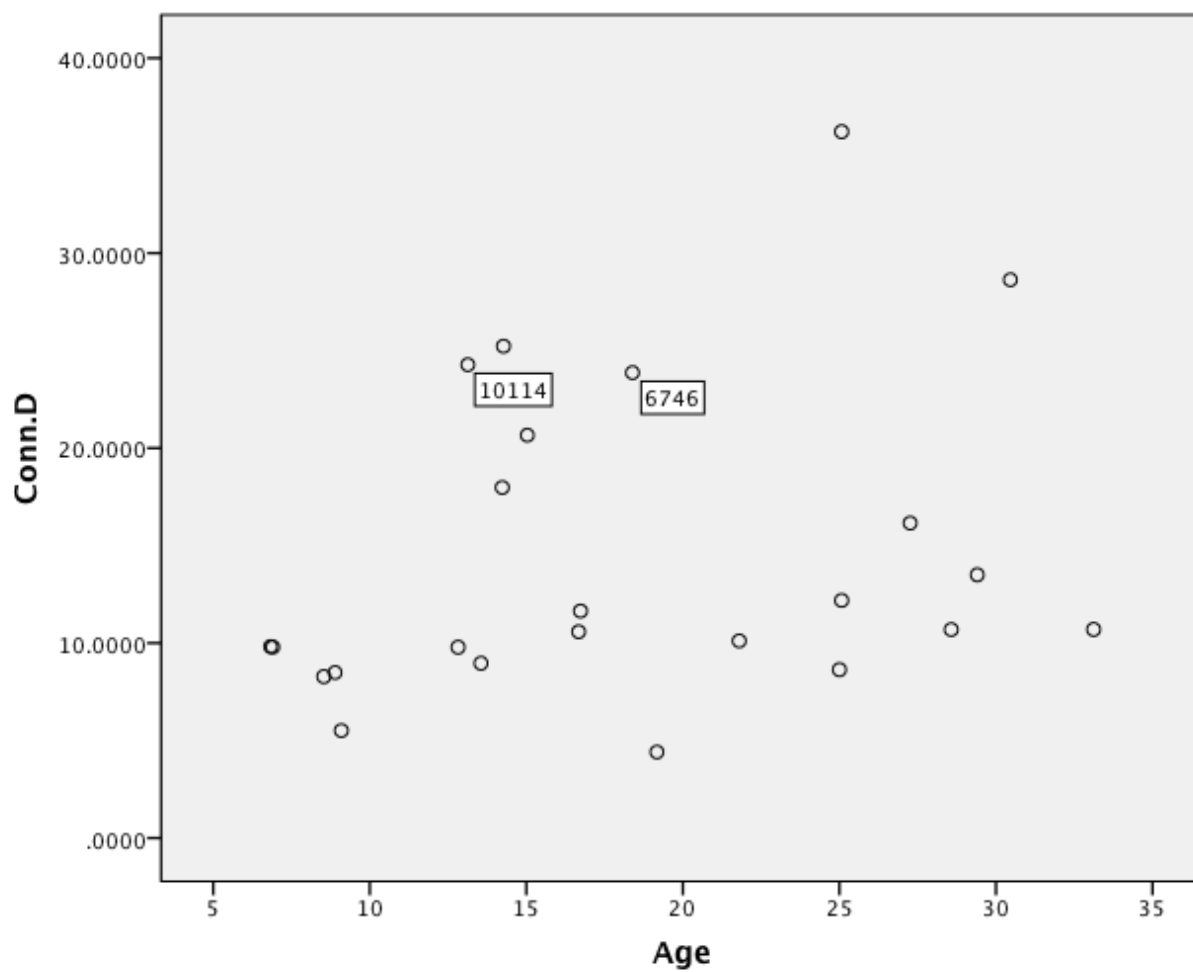
Fig. 4: Scatterplot showing age and Conn.D. Two females (6746, 10114) recently gave birth before their deaths and show unusually high Conn.D.
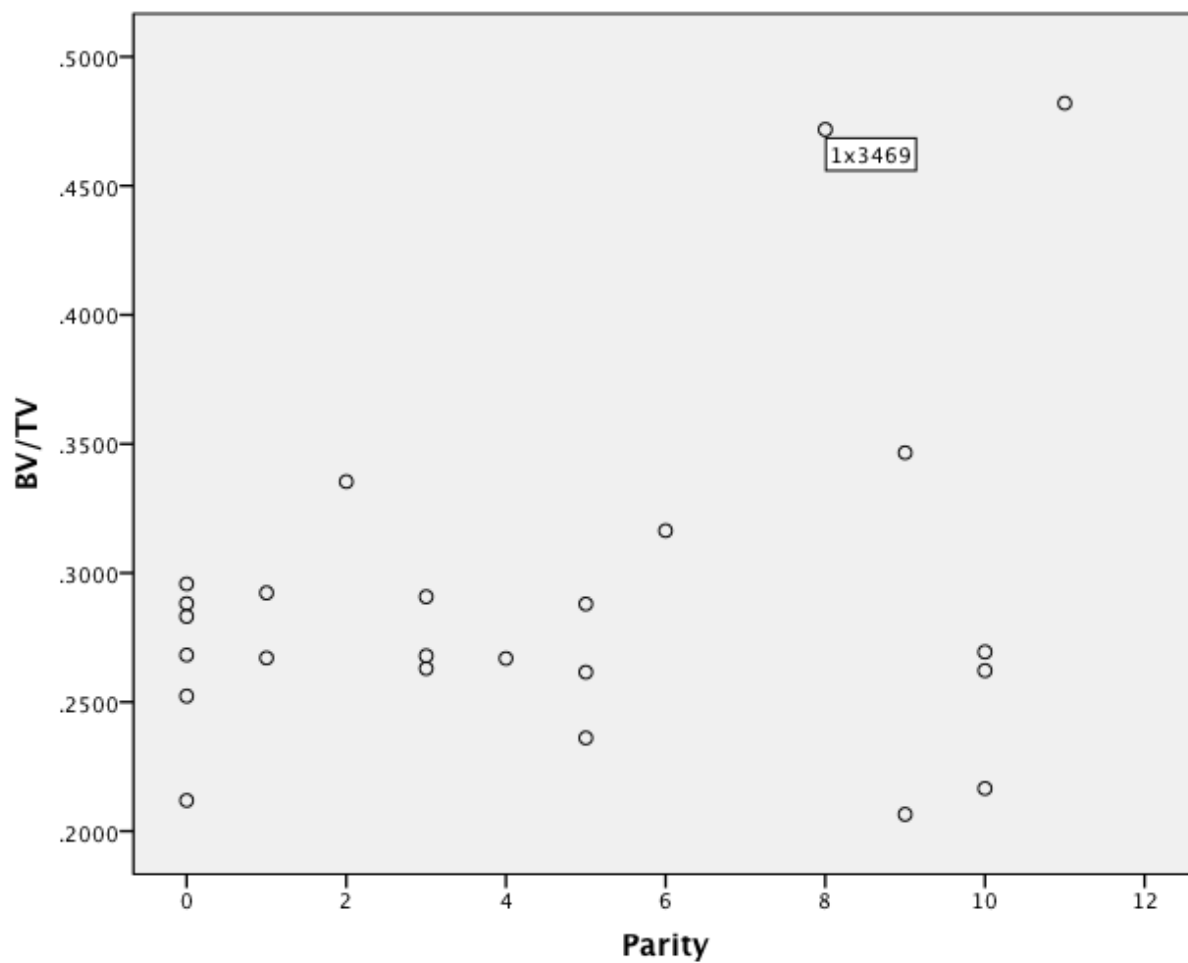
Fig. 5: Scatterplot showing BV/TV with respect to parity. Female 1x3469 shows unsually high BV/TV for her parity group.
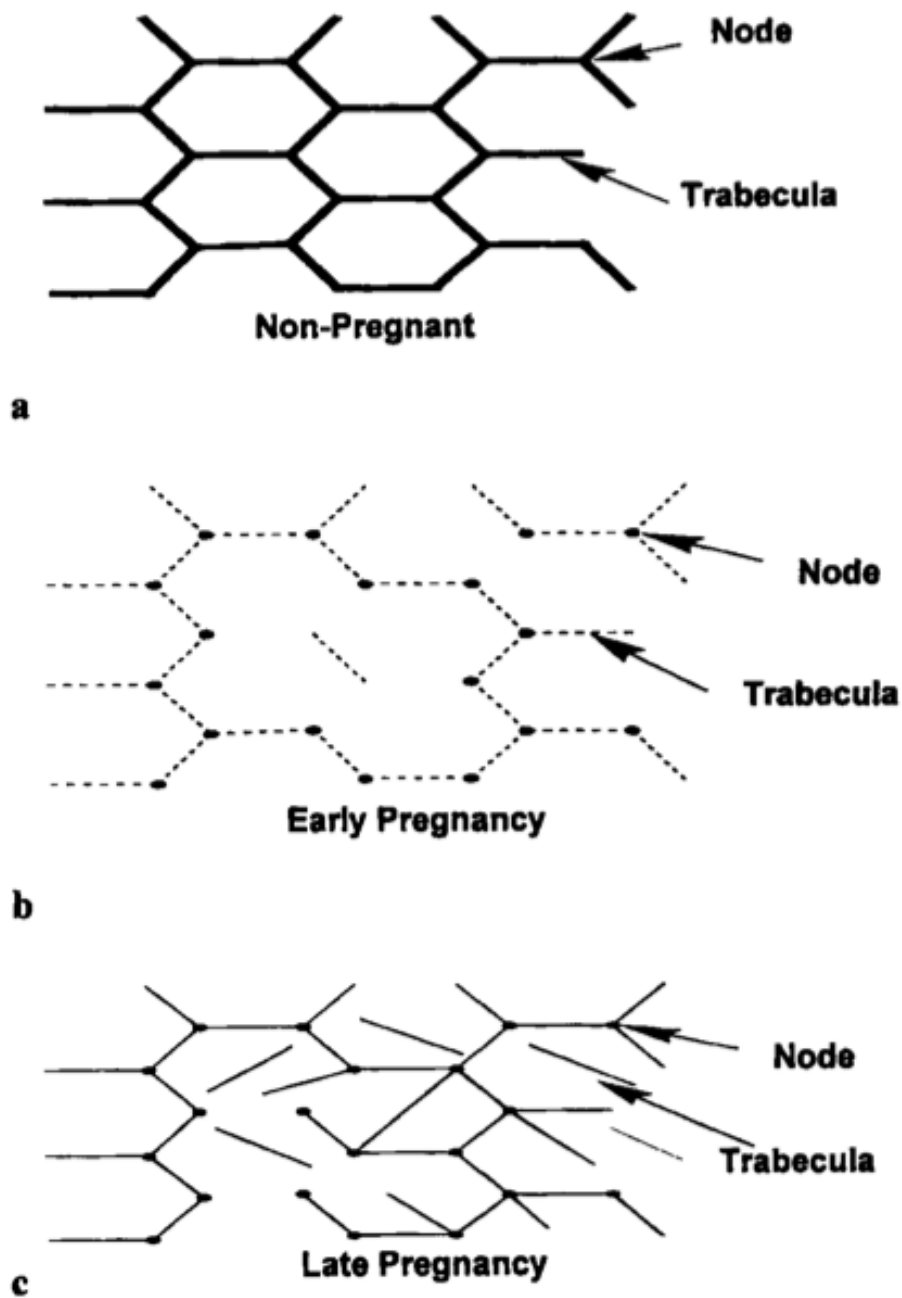
Fig. 6: Stylized drawings of the modification induced by pregnancy in cancellous bone structure, illustrating the trend away from a) relatively coarse interconnected network through b) trabecular attenuation and disconnection to c) a finer more complex network with increased struts (Shahtaheri et al., 1999).
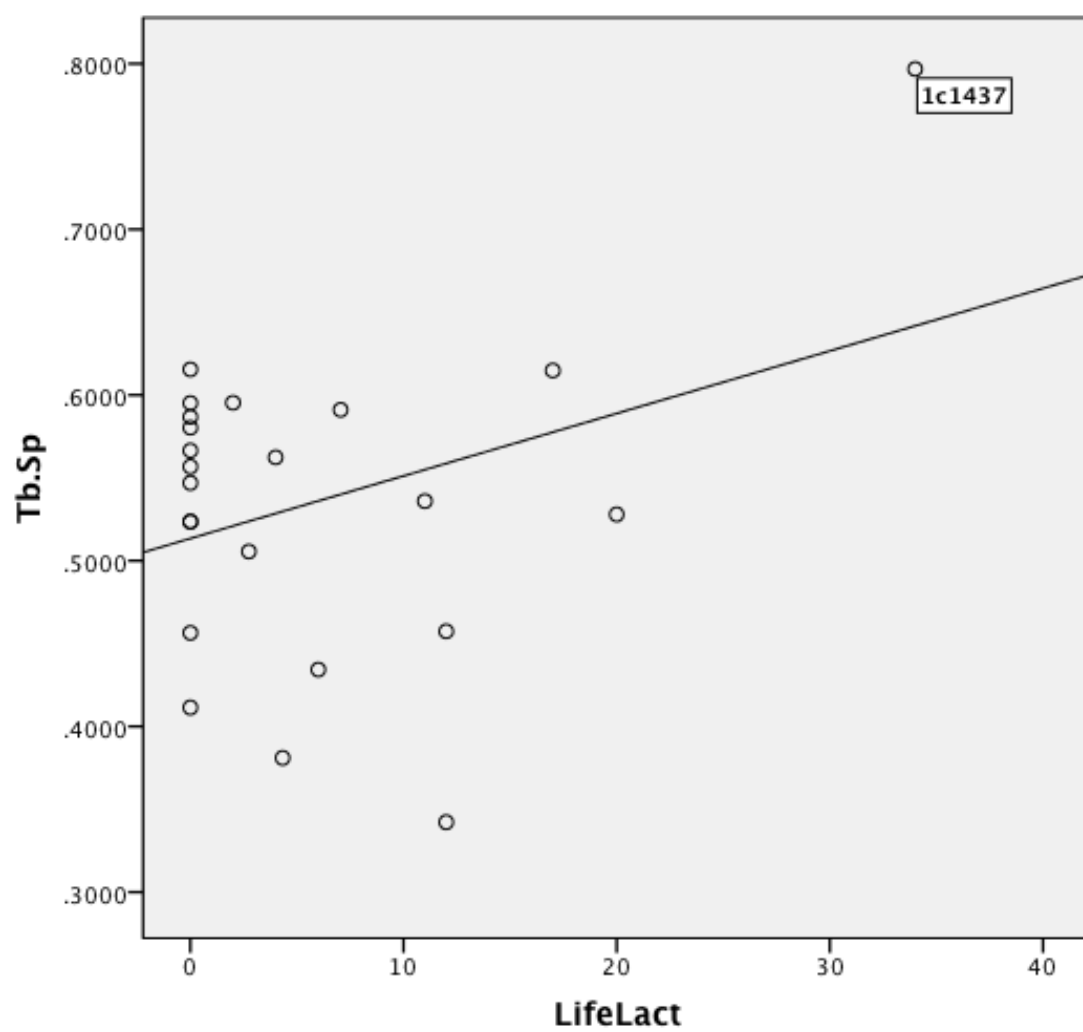
Fig. 7: Scatterplot showing increasing Tb.Sp with %LifeLact. Female 1c1437 has the highest %LifeLact (34%) and also the highest Tb.Sp.
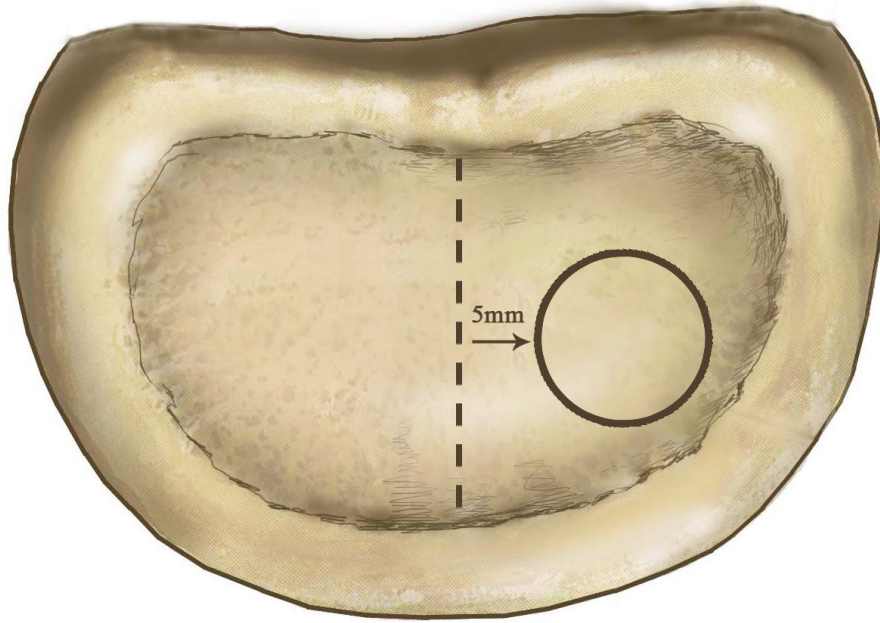
Fig. 8: Sketch showing the placement of the lumbar core on the superior surface of the L1 vertebra. The dashed line represents the midpoint of the vertebra. The core was taken 5mm lateral to the right.

Table 4: Summary of statistically significant correlations between trabecular architecture (L1 vertebral core) and cortical remodeling and cross-sectional area measurements (Humerus)

	r	P
ConnD & On.Ar	.483	.042
Tb.N & Ct.Ar	-.468	.050
Tb.Sp & %On.B	.497	.036
Tb.Sp & Tt.Ar	.568	.014
Tb.Sp & Ct.Ar	.527	.024

Results show r values and significant p-values (<.05) using the Pearson's correlation test

Chapter 7

Summary and Conclusions

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    This dissertation is broadly focused on how differences in age, sex, body weight, and maternal reproductive behaviors affect cortical and trabecular bone quality and quantity in aging baboons. The inherent complexity of the factors relating to overall bone tissue organization and structural integrity makes studying and interpreting human skeletal variation particularly challenging. Animal models (including rodents) have been essential in developing an understanding of the biological, behavioral, and genetic underpinnings of bone metabolism and remodeling. However, nonhuman primates (e.g. macaques and baboons) are more nearly ideal physiological and skeletal models of long-term bone turnover and microarchitecture. Baboons and humans share many cercopithecine traits, including a large body size, relatively long lifespan, and similar reproductive physiology and endocrinology (Brommage, 2001). Skeletally, baboons and humans also have the same organization of bone microarchitecture into Haversian systems (Jerome and Peterson, 2001), similar processes of remodeling and repair (Havill et al., 2013), as well as the same patterns of bone degeneration during aging (Aufdemorte et al., 1993; Cerroni et al., 2000; DeRousseau, 1985; Havill et al., 2003a; Kammerer et al., 1995; Havill et al., 2008, 2013).

    Chapter 4 tests the hypotheses that intracortical remodeling and cross-sectional area show significant differences by appendicular site (humerus vs. femur), and sex. Strong support was observed for each hypothesis. Site-specific patterns include significantly larger drifter osteon measurements (DOn.Ar, Dh.Ar) in the humerus and larger stationary osteon measurements (On.Ar, H.Ar) in the femur. The differences in osteon sizes may be related to the kinesiology and loading of hindlimb vs. forelimb mechanics during quadrupedal walking. Additionally, high %Po.Ar, high %On.B and low %C_A/T characterize the midshaft humerus as significantly more remodeled than the femur. The increased humeral to femoral %On.B in this study is unsurprising, and is an observation that has been reported in terrestrial quadrupedal primate species by several authors (Paine, 1994; Paine and Godfrey, 1997; McFarlin et al., 2008). In this study, much of the difference in appendicular site remodeling is explained by sex-specific patterns. At the midshaft humerus, males showed significantly larger On.Ar, H.Ar, DOn.Ar, and lower %On.B and OPD. This results in an interesting humerus-specific sexual dimorphism in osteon dynamics: males grow larger osteons and Haversian canals but have lower overall activation of remodeling whereas females have increased secondary osteon activity (OPD, %On.B) but smaller osteons and Haversian areas.   Smaller osteons in combination with increased OPD typically reflects a higher BMU activity, as the creation of new secondary osteons will take less time if they are smaller. This is relevant to females in that 1) a heightened activation rate could reflect a need to strengthen fatigue-damaged bone, or 2) a need to temporarily reduce bone mineralization (Pfeiffer et al., 2006). It is possible that female baboons have an increased need to strengthen fatigue-damaged humeral bone as a result of differences in physical activity rates and infant-rearing behaviors. Infant holding, carrying, and nursing behaviors could result in increased female humeral loading, likely from the added weight

associated with picking up and carrying young toward the front of the body, as well as the corresponding size increase in forearm musculature and periosteal apposition. Another possibility is that the increased fetal and neonatal calcium demands during multiple cycles of pregnancy and lactation cause reduced bone mineralization in the cortex, decreasing osteon and Haversian area sizes and increasing OPD. This result in combination with female-specific high %Po.Ar and low %C_A/T in both the midshaft humerus and femur led to the conclusion that the overall increase in cortical resorption and remodeling is likely related to the unique calcium demands of reproducing females.

Chapter 5 further explored the hypothesis that variation in female intracortical remodeling dynamics and cross-sectional area is related to reproductive history, particularly differences in parity, lactation length, and interbirth interval. An ANCOVA analysis (age held constant) shows significant differences between low and high parity groups in drifter osteon areas (DOn.Ar), with the high parity group showing larger osteon sizes. At the midshaft femur, IBIavg shows a positive relationship with On.Ar, suggesting that longer IBIavgs increase osteon size at this site. Overall, in baboons, reproductive history is associated with increases in osteon sizes at both the humerus and femur. These changes are likely related to the significantly elevated levels of estrogen seen during baboon pregnancy (Hendrickx and Dukelow, 1995). In female baboons, elevated estrogen is associated with increases in osteoblastic activity and decreases in osteoclastic activity in experimental contexts (Hotchkiss and Brommage, 2000; Lees and Jerome, 1998). It is likely that the increased osteoblast and reduced osteoclast activity could result in larger osteons, with a significant cumulative effect with high parity.

At the midshaft humerus, significant increases in %Po.Ar were seen in the high %LifeLact group, in comparison with the low %LifeLact group of females. Significant increases in intracortical porosity have also been observed in lactating mice, with only partial recovery of high lactation after a 28-day post-weaning period (Liu et al., 2012). In both our study and that of Liu et al., (2012), lactation is associated with increased resorption of the cortice and increased porosity. However, some repair of this porosity is seen after weaning. In another mouse study, as early as two weeks post-weaning significant active mineralization was present on eroded surfaces of cortical endosteum (Miller and Bowman, 2004), indicating a skeletal transition from resorption (during lactation) to formation (after weaning). In the present study, some evidence for this in baboons is also shown. Regression analyses showed that increased IBIavg is associated with a small but significant decrease in %Po.Ar at the midshaft humerus. Therefore, although the overall effect of high %LifeLact increases %Po.Ar in baboons, some repair of this occurs with longer interbirth intervals between successive offspring.

Significant effects of reproductive history are also seen in cross-sectional area at the humerus and femur. Regressions and ANCOVAs revealed that Tt.Ar is significantly increased by both high %LifeLact and high parity at the midshaft humerus, and high %LifeLact at the femur. Both weight and increased %LifeLact had anabolic effects on Tt.Ar and Ct.Ar at the midshaft humerus, particularly in the old adult group (ages 19-33). Together, the larger Tt.Ar and Ct.Ar in females with high %LifeLact and parity indicate significant periosteal apposition in these groups. The increased biomechanical strain associated with carrying suckling infants, in addition to the increased maternal body weight during pregnancy, may have stimulatory anabolic effects on the periosteum of cortical bone and may account for the increased total and cortical area

measurements seen in these groups. Similar appositional effects of increasing parity on cortical thickness were seen in Bowden et al., (1979).   However, measurements of cortical area thickness do not reveal information about the porosity of intracortical bone, and "true" measurements of cortical area may be significantly lowered after accounting for the percentage of void areas inside the cortex. In this study, although the Tt.Ar and Ct.Ar are larger with increased %LifeLact, the total percentage of adjusted cortical area (%C$_A$/T) after accounting for the porosity is lower females with high %LifeLact. This suggests that the behavioral aspects of infant-rearing associated with long periods of juvenile care and nursing increase the outer cortex (periosteum) of the humerus but low estrogen levels and increased osteoclast activity during breastfeeding decrease the total amount of endosteal bone inside the cortice through increased porosity. This pattern of pregnancy-related increases in cross-sectional area (Miller et al., 1986), followed by significant resorption of the endosteum during lactation (Miller and Bowman, 2004) is also observed in rats. Lactation-induced increased endocortical resorption without negative changes to cross-sectional geometry has also been observed in humans. Laskey et al. (2011) observed significant BMD decreases in femoral shaft cortical bone mass in lactating women. Despite the lowered cortical BMD, no changes to femur cross-sectional area were seen and the authors interpreted the mineral losses as having occurred internally through porosity of the endosteum (Laskey et al., 2011).   In the present study, ANCOVAs revealed significant increases in humerus medullary area (Es.Ar) with respect to increasing parity, further highlighting cortical endosteal bone as significantly reduced by increased cycles of pregnancy and lactation. Significantly larger medullary areas have also been observed in rat females who have undergone multiple pregnancies, compared with younger or older nulliparous groups (Miller and Bowman, 2004).

Chapter 6 investigated the effects of variation in female reproductive history on trabecular quantity and micro-architecture. In contrast to the large sample sizes analyzed in Chapters 4 and 5, a small sample of females was analyzed in Chapter 6, due to the cost and time constraints of micro-CT analysis. Although none of the regression analyses and t-tests showed significance, scatterplots revealed some interesting trends. Two females who had recently given birth before their deaths showed unusually high Tb.N, Conn.D and low Tb.Sp. These trabecular bone changes were also observed in iliac biopsies taken from full-term women at the time of scheduled c-section (Shahtaheri et al., 1999). At the end of pregnancy, the appearance of new intratrabecular resorption cavities transformed previously thickened trabecular plates into multiple thinner ones, increasing both the Tb.N and Conn.D (Shahtaheri et al., 1999).

Analyses here also revealed a potentially interesting relationship between lactational history and Tb.Sp, Tb.N, and ConnD. A female with a history of very extended lactation (%LifeLact=37%) is a statistical outlier in trabecular architecture in several respects. This outlier female (1c1437) has abnormally high Tb.Sp, and abnormally low Tb.N and ConnD. Interestingly, her Tb.Th is the normal range, so the issue is not related to significantly thinned trabeculae, but rather the lower total number of trabeculae and larger spaces between them. Confounding effects of aging are not an issue in this case, as she is only 19 years old and the older individuals in the sample (as old as 33 years) have very different results in Tb.Sp, Tb.N and ConnD. These results are similar to those seen by Liu et al., (2012), where lactation in mice was associated with marked deterioration in plate number (pTb.N), as well decreases in plate-rod and plate-plate junction densities (P-R and P-P Junc.D). Even after weaning a significantly lowered

P-R ratio (similar to ConnD) and pTb.N remained, suggesting long-standing changes to micro-architecture as a result of lactation. The results of this study, as well as those of Liu et al., (2012) suggest that some aspects of trabecular architecture may never recover to values comparable to pre-lactation levels. Although short-term pregnancy-related changes may include increased Tb.N and Conn.D, the large resorption of cancellous bone that occurs during multiple cycles of long lactation appears to deteriorate these "new" struts, leaving behind large spaces, a reduced number of trabeculae, and decreased connectivity.   However, both these short and long-term alterations are based on a small number (n=3) of outliers and need to be more rigorously tested in a larger sample group.

Conclusion and future work

According to evolutionary theory, females in an iteroparous species that produce multiple offspring over time (e.g. monkeys and humans) would have selectively evolved mechanisms to maintain the skeleton over an entire lifetime of reproduction (Ott et al., 1999; Wysolmerski, 2002) to increase reproductive success and optimal offspring survival. If evolution favored mechanisms that conserve and repair skeletal tissues during these reproductive periods, there should be no or little detrimental effect to the skeleton in the long term. This body of work tests the theory that differences in reproductive behaviors will have significant long-term effects on female cortical and trabecular bone quality and quantity. In humans, the relationships of parity and lactation histories with respect to long-term bone health are not uniformly established, with some studies showing positive (e.g. Hiller et al., 2003; Cure-Cure et al., 2002) and others showing negative effects (e.g. Canal-Macias et al., 2012; Schnatz et al., 2010). Some of this conflict is explained by the results of the present study, which shows that parity, %LifeLact, and IBIavg all exhibit different effects by site and by microarchitectural or cross-sectional area variable studied. At the midshaft humerus detrimental effects of parity and high %LifeLact are seen in significantly lower %C/T and %C_A/T at both the femur and humerus in high parity females. %Po.Ar is also significantly increased by high parity at the midshaft humerus. However, positive effects of high %LifeLact are seen in significant increases in Tt.Ar at both humerus and femur, and in increased Ct.Ar at the humerus. Miller and Bowman, (2004) hypothesize that the inverse reproductive-specific relationship of periosteal deposition with endosteal resorption may have an adaptive mechanical significance. Endocortical resorption results in a decrease in mechanical strength of cortical bone (Currey and Hughes, 1973; Vadja et al., 2001) and the increases in periosteal apposition and overall cross-sectional area may help protect the maternal skeleton from fracture during this catabolic period (Miller and Bowman, 2004). As long as periosteal apposition continues, the effects of increased intracortical porosity should not have detrimental effects on overall cortical bone health and fracture. Therefore, the overall effects of high parity and lactation on appendicular cortical bone health seen here in the humerus and femur are theoretically neutral.

In the same vein, although analysis of an outlier female in Chapter 6 showed that high %LifeLact is associated with increased Tb.Sp and decreased Tb.N and ConnD, her Tb.Th and BV/TV are in the normal range. Because BV/TV (and degree of mineralization) has the most significant effects on bone strength and stiffness (Burr, 2002; Wang et al., 2002), it is unclear if this outlier female's increased Tb.Sp, decreased Tb.N and ConnD put her at a mechanical

disadvantage. Therefore, the long-term effects of reproductive differences at the lumbar site studied here also appear to be neutral.

Future work investigating both cortical and trabecular micro-architectural changes with reproductive history should include mechanical testing of specimens to clarify how these combinations of microstructural alterations affect bone fragility and fracture risk. In particular, assessment of the correlations between intracortical remodeling differences and cross-sectional geometry would further clarify whether the increased periosteal apposition in combination with endocortical resorption has significant impacts on cortical bone mechanical properties in females with extended lactation histories. The result of lactation history in positively affecting cross-sectional area at both sites should also be further clarified though detailed investigation of behavioral, mechanical loading, and possible dietary changes that occur in SNPRC baboons during the nursing period. Lastly, experimental work testing the short-term changes of pregnancy and lactation on skeletal microstructural processes of resorption and repair is typically carried out on females without any prior pregnancies or lactational history. Experimental studies observing short-term bone changes should further clarify how females with high parities differ from females with no prior gestational history during and after pregnancy to see if bone responds differently to the stresses of reproduction over the lifespan.

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