The effects of dietary and skeletal calcium availability on reproductive performance of mammals

by

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Abstract

Investment in offspring production often requires the mobilization of endogenous resources, a strategy which may negatively impact maternal condition. In mammals, skeletal ossification in growing offspring requires considerable calcium investment by the mothers, and bone loss has been described in several species as a means of supporting this demand. Although bone loss can have adverse effects, its potential role in a reproductive trade-off has not been addressed. Using white-footed mice (*Peromyscus leucopus*) and domestic swine (*Sus scrofa*) I investigated the following questions: 1. Does dietary calcium intake exacerbate bone loss as a cost of reproduction? 2. How do mothers allocate calcium in response to dietary availability? 3. How does offspring production influence bone metabolism independent of diet? 4. How do mothers respond to calcium availability to maximize lifetime reproductive success? I characterized changes in maternal skeletal condition, variation in pup characteristics and overall reproductive output of the mothers. Bone characteristics were quantified via dual energy x-ray absorptiometry, bone marker assays, 3-point flexural tests, micro-computed tomography, and mineral composition (using both ashing and inductively-coupled plasma spectrophotometry). To assess the degree of calcium allocated to offspring, I measured bone morphology and mineral composition of pups. Reproductive output was characterized by litter size, individual offspring and litter mass, sex ratio, and total number of offspring produced. Under low dietary calcium conditions, females reproduced at a cost to their own skeleton. This cost was long term, as mothers that had consumed a low-calcium diet throughout their lives exhibited an overall

reduction in bone volume. Offspring production also affected bone metabolic activity independent of maternal calcium intake, and multiparous females responded less drastically to offspring demands relative to primiparous or generally reproductively inexperienced females. Over the course of their lifetime, mothers on a low-calcium produced generally smaller litter sizes as well as female-biased litters, indicating that they had indeed responded to dietary calcium availability and had adjusted investment/allocation accordingly. Thus, I show that calcium availability and bone loss can provide a tractable means for evaluating reproductive trade-offs in vertebrates.

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Introduction

In order to successfully reproduce, female mammals must allocate a substantial amount of resources into the creation and development of their progeny. Such resources might otherwise be used to support somatic functions; thus, mothers may trade off their own physical condition and offspring production (Roff, 1992; Stearns, 1992). Investment of resources into offspring production may result in reduced survival or reduced future reproductive success and therefore must be balanced against these costs to maximize lifetime reproductive success (Charnov, 1997; Clutton-Brock, 1984; Lack, 1947, 1948; Pontier et al., 1993; Roff, 1992; Stearns, 1992; Williams, 1966).

Classically, reproductive trade-offs have been investigated within the context of energy use and allocation (e.g. Boutin, 1990; Speakman, 2008) Reproduction is indeed an energetically costly function, although other resources are also critical to the successful production of progeny. For vertebrates, a substantial amount of calcium must be transferred to developing young to support the rapid mineralization of their osseous skeletons. In mammals, calcium is transferred via the placenta during gestation and via the milk during lactation. The calcium demands of reproduction frequently stimulates mobilization of bone mineral from the maternal skeleton, and bone loss during gestation and lactation is common in most mammals studied to date (Benzie et al., 1955; Boelter and Greenberg, 1943; Brommage, 1989; Hood et al., 2006; Kalkwarf and Specker, 2002; Lees et al., 1998; Liesegang et al., 2007; Ott et al., 1999; Prentice, 2000; Speakman, 2008; Wysolmerski, 2010; Zeni et al., 1999). The degree that bone mineral is

resorbed from the skeleton has been shown to be influenced by maternal calcium intake – more bone is mobilized when dietary calcium intake is low – suggesting that females draw on skeletal reserves to compensate for deficiencies in the diet (Boelter and Greenberg, 1943; Brommage and DeLuca, 1985; Peng et al., 1988).

Bone strength decreases as mineral is removed from bone (Broe et al., 2000; Marshall et al., 1996; Reilly and Burstein, 1975), which results in an increased fracture risk. For a wild animal, a broken bone may impair foraging or predator avoidance, or may result in an infection, thus, increased fracture risk can be associated with a decreased likelihood of survival. Additionally, bone loss may result in a diminished mineral reserve available for investment into future reproductive efforts. Therefore, bone mobilization can be viewed as a cost of reproduction, affecting maternal skeletal condition and endogenous resource availability. Given this cost, the extent that mineral is mobilized from bone should presumably be balanced against the benefit of offspring production.

Since maternal transfer of considerable amounts of calcium is required to facilitate offspring production, I hypothesized that female mammals trade off skeletal condition and offspring production. Based on this general hypothesis, I investigated the following: 1. the influence of low dietary calcium intake on maternal skeletal condition and reproductive output, 2. the influence of maternal calcium intake on the degree of mineral allocated to the production of offspring, 3. the influence that production of offspring has on maternal bone metabolic activity, and 4. adjustments in reproductive output relative to dietary calcium availability.

I approached this by using reproductive female white-footed mice (*Peromyscus leucopus*) and domestic swine (*Sus scrofa*). I manipulated the calcium intake of white-footed mice and assessed maternal skeletal condition, reproductive performance, and mineral allocation to

offspring production. For the swine, intake was held constant to assess the effects of offspring production and maternal mass and parity on maternal bone metabolic activity. I predicted that the production of offspring is associated with a reduction in maternal skeletal condition, and that this relationship is influenced by exogenous calcium availability. Therefore, mothers that produce more offspring should mobilize more bone, and should adjust reproductive output in response to dietary calcium availability.

This work illustrates a novel means with which to investigate classic models of reproductive trade-offs, and provides insight into how the availability of specific nutrients may influence how an animal may invest in reproduction.

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I. Bone loss as a physiological cost of reproduction in white-footed mice

Abstract

Investment in offspring production often requires the mobilization of endogenous resources, a strategy which may negatively impact maternal condition. In mammals, skeletal ossification in growing offspring requires considerable calcium investment by the mothers, and bone loss has been described in several species as a means of supporting this demand. Although bone loss can have adverse effects, its potential role in a reproductive trade-off has not been addressed. Using white-footed mice (*Peromyscus leucopus*), we tested the effect of dietary calcium availability on maternal skeletal condition during reproduction to assess if calcium availability drives a trade-off between maternal skeletal condition and offspring production. We provided mice with a low-calcium or standard diet and monitored reproductive output along with changes in bone mineral density (BMD) and bone metabolism throughout reproduction. Reproductive females on the low-calcium diet showed a significant reduction in BMD and a significant difference in bone formative activity relative to reproductive females consuming the standard diet and non-reproductive mice consuming the low-calcium diet. Reproductive performance was not negatively affected by low calcium intake. Our results suggest that when dietary calcium is limited white-footed mice reproduce at the expense of their skeletal condition.

Introduction

To reproduce, organisms must be able to acquire and utilize materials essential to sustain production, and in many cases, the development of their progeny. To meet the elevated nutritional demands of reproduction, a mother may be faced with a physiological trade-off, where investment in offspring may come at the expense of her own condition (Roff, 1992; Stearns, 1992). Supporting reproduction over self-maintenance can negatively impact a parent's probability of survival and future reproductive value. Thus, allocating resources to the current reproductive effort is expected to be balanced against its impact on lifetime reproductive success (Charnov, 1997; Stearns, 1992; Williams, 1966). This physiological trade-off has been the focus of numerous empirical and theoretical studies since it was first articulated by Williams (1966) in his refinement of Lack's optimal clutch size hypothesis. The majority of studies characterizing the trade-off between investment in offspring and self-maintenance focus on energy utilization and investment; however other nutrients may also be critical to this balancing act.

In mammals, offspring production requires substantial calcium transfer from mother to offspring to facilitate the mineralization of the offspring's skeleton. To support this demand, mothers may supplement dietary calcium with calcium mobilized from their own skeleton, which contains approximately 99% of the body's calcium (Bessey et al., 1935). Most mammals that have been studied experience bone loss resulting from elevated calcium mobilization over the course of gestation and lactation (Benzie et al., 1955; Brommage, 1989; Hood et al., 2006; Liesegang et al., 2007; Ott et al., 1999; Wysolmerski, 2010). In some species, bone loss during reproduction can be substantial, and low calcium intake can exacerbate this loss (Boelter and Greenberg, 1943; Brommage and DeLuca, 1985). For example, reproductive rats lose up to 28% of the bone mineral from their femurs on a 0.4% calcium diet, and up to 53% of bone mineral on

a 0.1% calcium diet (Peng et al., 1988). Thus, bone resorptive activity can serve as a compensatory mechanism for low availability of dietary calcium. Reduced bone mineral density corresponds with diminished bone strength (Broe et al., 2000; Marshall et al., 1996; Reilly and Burstein, 1975) and consequently, increased risk of sustaining a fracture.

Research that addresses bone loss during reproduction has been primarily conducted within a biomedical or agricultural context using domesticated or laboratory animals; however this phenomenon can feasibly have ecological implications. For a wild animal, a fractured bone can hinder foraging ability and predator avoidance, and as a consequence reduce the animal's probability of survival and future reproduction. Loss of bone also means loss of calcium reserves, which could limit the amount of calcium available to support subsequent reproductive bouts. Thus, utilization of the skeleton as source of calcium can be viewed as a cost of reproduction (Speakman, 2008), with dietary calcium availability driving a trade-off between maternal skeletal maintenance (i.e. survival) and allocation of calcium to offspring (i.e. reproduction).

Since calcium is elemental and essentially stored in one type of tissue, we propose that evaluating calcium acquisition, utilization and allocation provides a tractable means for studying reproductive trade-offs in vertebrates in contrast to energy, which is complicated by derivation from any proportion of the three macronutrients and storage in several tissues types within the body. We tested the hypothesis that dietary calcium availability influences the mobilization of maternal endogenous calcium stores during reproduction in white-footed mice (*Peromyscus leucopus*). At the fast end of the life-history continuum of mammals, white-footed mice are small (~30 g), reproduce frequently, and have relative short life spans. Thus, we expect that the costs incurred during reproduction in this species are high (Speakman, 2008), and potentially more

detectable, than in larger species with lower reproductive costs. We monitored bone mineral density and bone metabolism over the course of reproduction for mice fed a low-calcium or a standard diet and examined the relationship between diet, reproductive output, and patterns of bone mobilization by mothers.

Materials and Methods

We obtained 24 virgin white-footed mice (16 females, 8 males) from the Peromyscus Genetic Stock Center at the University South Carolina (Columbia, SC, USA) and transferred them to the lab animal facility at Auburn University (Auburn, AL USA). Mice were of similar age, 12 - 15 weeks old, at the beginning of the experiment. Females were randomly paired and housed in polypropylene 29 x 19 x 12.5 cm rodent cages (Lab Products, Inc., Seaford, DE USA) with *ad libitum* access to food and water. All mice were maintained at 25^{o}C on a 16:8 light cycle. After a 4 week acclimation period, one male was introduced to each female cage and then removed 21 days later. Females were monitored daily for evidence of parturition, and pups were counted 48 h post-partum and monitored daily to assess survival.

Mice received one of two custom manufactured diets (Harlan Teklad, Madison, WI USA) from the day of male introduction until after all offspring had been weaned (15 July 2008 – 18 September 2008). Half of the mice (8 females, 4 males) received a standard diet that contained 0.6% calcium, as recommended by the Peromyscus Genetic Stock Center for breeding mice. The other half (8 females, 4 males) received a low-calcium diet that contained 0.02% calcium. Diets were isocaloric and varied only in calcium carbonate content (Table 1). After all pups were weaned, mice that had consumed the low-calcium diet were provided with the standard diet which served as a rescue diet to counteract potential consequences of experimentally induced

calcium deficiency. We estimated mean daily food intake by determining the mass of food consumed by the mice in each box over 7 days, and dividing that mass by 7 days and the number of mice present. We excluded food intake data from all statistical comparisons for boxes housing pups greater than 20 days old, the point at which pups were observed consuming solid food (CMBS personal observation).

We measured bone mineral density (BMD) of the right and left femurs and tibias, and of the lumbar vertebrae (L4-L6) with dual-energy X-ray absorptiometry (DXA; Lunar PIXImus densitometer, Lunar Corporation, Madison, WI USA). Mice were transported to the Small Animal Bone Phenotyping Laboratory at University of Alabama at Birmingham on four occasions to complete the DXA scans 2 days prior to breeding, during mid-lactation (15-23 days post-partum), post-lactation (> 30 days post-partum), and 25 days after all animals had been returned to the standard diet (hereafter rescue). The number of DXA scans was limited by the availability of the instrument and a desire to minimize the stress of transportation on the mice. Breeding was not synchronous, as such, not all females were available for scanning at all 4 time periods. BMD of right and left femurs and tibias were averaged to provide a mean BMD for each bone.

We monitored bone metabolism by quantifying serum concentrations of alkaline phosphatase (ALP) and pyridinoline (PYD) crosslinks. ALP is produced in several tissues of the body, including the bone, liver, kidneys, and intestine, and has been identified as playing a role in bone mineralization (Whyte, 1994). Despite its wide occurrence, ALP concentrations have been shown to be closely correlated with bone turnover and bone-specific alkaline phosphatase (BAP) concentrations (Katagiri et al., 1995; Mohamadnia et al., 2007; Urena et al., 1996). Although the BAP assays are more specific than ALP, we were limited to the use of ALP

because commercially available assays for BAP did not exhibit reactivity with white-footed mouse serum. PYD crosslinks type I collagen in bone and is released into the circulation during bone resorption (Eastell et al., 1997). Bone is constantly undergoing remodeling, in which resorptive and formative activities are balanced. When this process becomes uncoupled, net bone loss or growth can occur. Thus, monitoring the relative concentrations of these two products can provide insight into the degree of bone loss or growth that may be occurring within an animal at a given time. Serial dilutions of white-footed mouse serum showed evidence of reactivity and undiluted serum produced ALP values similar to those reported for laboratory mice (unpublished data). Starting approximately 2 weeks prior to breeding, we collected serum samples weekly from all mice between 13:00 and 15:00 h; sampling continued until the last dam had weaned her pups. Whole blood was collected into capillary tubes after lancing the lateral saphenous vein with a 26 gauge needle. Serum was immediately separated via centrifugation and then stored at -80° C until analysis. Blood was not collected during the first 7 days postpartum to minimize stress on the females. ALP was measured using a colorimetric kinetic assay (QuantiChrom™ Alkaline Phosphatase Assay Kit, BioAssay Systems, Hayward, CA USA) and serum PYD was measured using an EIA (MicroVue Serum PYD, Quidel® Corporation, San Diego, CA USA).

Data Analyses

Mean BMD of femurs and tibias, BMD of lumbar vertebrae and log-transformed serum concentrations of ALP and PYD throughout the reproductive period were compared between diets and with the reproductive status in female mice, and between reproductive and non-reproductive mice (non-reproductive mice included males), using a repeated-measures ANOVA with an AR1 covariance structure (PROC MIXED). Individual mouse identity was included as a

random effect and time as the repeated variable. When there was no significant interaction between diet and time or between reproductive status and time, these variables were tested separately to compare overall values of the dependent variables. Covariates and models used are shown in Table 2. Females were considered reproductive when they were pregnant or lactating, thus females that produced pups that died within 48 h post parturition were considered to be reproductive during gestation, but were eliminated from analyses of reproductive females during lactation. Log-transformed individual daily food intake data was compared between diets and reproductive status of females using a general linear model (PROC GLM). Litter size was compared between treatment groups using a t-test and results were tested using a randomization test to account for sample size (Resampling program; David C. Howell, University of Vermont, pers. comm.). All other statistical analyses were conducted in SAS 9.1 (SAS Institute; Cary, NC USA).

Results

Reproductive Output

Four females on the standard diet and four females on the low-calcium diet produced litters that survived past 48 h post-partum. One female consuming the low-calcium diet did not give birth and did not appear to be pregnant, and pup death within 48 h post-partum only occurred in females on the low-calcium diet, in which three complete litters died and were partially or totally consumed before they could be accurately quantified. Litter size was statistically similar between dietary groups, although females on the low-calcium diet tended to produce more offspring (Standard: $\bar{x}$ = 2.50 ± 0.50 SE pups, Low: $\bar{x}$ = 3.75 ± 0.25 SE; $t_6 = 2.24$, $P = 0.067$).

Food Intake

Food intake increased over time for reproductive females on both diets (Fig. 1) but there was no significant difference between diets nor was there an interaction between time and diet for reproductive females ($F_{1,34} = 0.51$, $P = 0.480$). There was no significant difference in food intake over time for non-reproductive mice on either diet (Low: $F_{1,38} = 0.58$, $P = 0.451$; Standard: $F_{1,32} = 0.01$, $P = 0.909$), nor was there a significant interaction between diet and time for non-reproductive mice (Fig. 1).

Bone Mineral Density

Change in BMD of tibia and lumbar vertebrae over time differed significantly between diets for reproductive females, with females on the low-calcium diet demonstrating a greater reduction in BMD than females on the standard diet (Tibia: $F_{3,22} = 3.67$, $P = 0.028$; Vertebrae: $F_{3,22} = 4.14$, $P = 0.018$; Fig. 2; Table 2). Change in femur BMD reflected a similar trend ($F_{3,22} = 3.04$, $P = 0.0506$; Fig. 2; Table 2). There was no significant difference in BMD of femur, tibia or lumbar vertebrae over time between reproductive and non-reproductive mice consuming the standard diet (Femur: $F_{3,18} = 0.88$, $P = 0.471$; Tibia: $F_{3,18} = 0.670$ $P = 0.580$; Vertebrae: $F_{3,18} = 2.27$, $P = 0.115$; Table 2) or between reproductive and non-reproductive females consuming the low-calcium diet (Femur: $F_{3,24} = 1.74$, $P = 0.187$; Tibia: $F_{3,24} = 2.03$ $P = 0.137$; Vertebrae: $F_{3,24} = 0.62$, $P = 0.610$; Table 2). When time was removed from the model, overall BMD of all 3 bones differed significantly with reproductive status of the mice consuming the low-calcium diet (Femur: $F_{1,9} = 6.93$, $P = 0.027$; Tibia: $F_{1,9} = 6.56$, $P = 0.031$; Vertebrae: $F_{1,9} = 6.99$, $P = 0.027$; Table 2). Overall femur BMD differed significantly with reproductive status of mice consuming the standard diet ($F_{1,7} = 6.75$, $P = 0.036$; Table 2) and BMD of tibia lumbar vertebrae showed a

similar trend (Tibia: $F_{1,7} = 5.14$, $P = 0.058$; Vertebrae: $F_{1,7} = 5.59$, $P = 0.050$; Table 2). BMD changed significantly over time for mice on both diets ($P \leq 0.006$ for all comparisons; Table 2), with the exception of femur BMD of mice consuming the low-calcium diet ($F_{3,24} = 2.86$, $P = 0.058$; Table 2).

Bone Markers

Serum concentrations of ALP did not differ significantly over time between diets for reproductive females ($F_{5,11} = 1.28$, $P = 0.340$; Table 2) or with reproductive status of mice on either the low-calcium or standard diet (Low: $F_{5,17} = 0.870$, $P = 0.524$; Standard: $F_{4,11} = 1.83$, $P = 0.194$; Fig. 3; Table 2). There was also no significant difference over time for reproductive or non-reproductive mice ($P \geq 0.096$ for all comparisons; Table 2). When time was removed from the model, overall serum concentrations of ALP were significantly higher for reproductive females on the low-calcium diet compared to reproductive females on the standard diet ($F_{1,6} = 16.82$, $P = 0.006$; Table 2) and for reproductive females on the low-calcium diet compared to males and non-reproductive females on the low-calcium diet ($F_{1,7} = 18.88$, $P = 0.003$; Table 2). There was no difference in serum ALP concentrations between reproductive females and non-reproductive mice (including males) in the standard diet ($F_{1,10} = 0.66$, $P = 0.434$; Table 2).

Serum concentrations of PYD did not differ between diets over time for reproductive females ($F_{5,17} = 1.08$, $P = 0.408$; Table 2) or with the reproductive status of mice on either the low-calcium or standard diets (Low: $F_{5,21} = 0.500$, $P = 0.776$; Standard: $F_{6,21} = 0.58$, $P = 0.740$; Fig. 4; Table 2). There was also no significant difference in overall serum concentrations of PYD over time or between diets for reproductive or non-reproductive mice ($P \geq 0.434$ for all comparisons; Table 2).

Discussion

Our results demonstrate that the availability of calcium in the diet influences bone loss, and that this bone loss can be interpreted as a physiological cost of reproduction in white-footed mice. As such, calcium availability may serve as a driving factor in the trade-off between maintenance of maternal bone and offspring production in this and other small species of mammals. The amount of calcium ingested affected reproductive performance, and dietary calcium, the production of offspring and maternal skeletal condition interacted in a manner that fits theoretically proposed reproductive strategies.

Females from each dietary group successfully produced litters and thus, it is clear that dietary calcium restriction did not prevent reproduction in white-footed mice. Indeed, our results suggest that females consuming the low-calcium diet tended to produce more offspring than those on the standard diet. The death and consumption of pups within 48-h postpartum only occurred in the low-calcium group. Thus, it is feasible that some females on the low-calcium diet were unable to allocate sufficient calcium to support lactation, which is generally considered to be the period of highest calcium demand in mammals. It is also possible that the consumption of pups would have allowed females to recover some of the bone mineral lost to their offspring (Hood, submitted). However, this interpretation should be considered with caution because death of pups immediately following parturition is relatively common in rodents.

Reproductive females on the low-calcium diet did appear to consume more food on a daily basis than reproductive females on the standard diet (Fig. 1). Given the disparate amount of calcium present in the two diets, females on the low-calcium diet would have had to consume 30 times more food in order to ingest the amount of calcium available in the standard diet. Thus the slight increase food intake observed in females on the low-calcium diet would have had a

negligible effect on calcium available for offspring production. The observed increase in food consumption as the reproductive bout progressed can be attributed to elevated caloric and nutrient demands associated with pregnancy and lactation.

Females that consumed the low-calcium diet and produced successful litters experienced a considerable reduction in BMD relative to both reproductive females on the standard diet and non-reproductive females on the low-calcium diet. This suggests that bone mobilization is compensating for reduced intake of calcium thus helping the female to meet calcium demands of reproduction. Diet alone did not affect BMD in non-reproductive mice, underscoring the substantial calcium demand associated with reproduction in mammals.

Serum concentrations of PYD over the reproductive bout suggest that bone resorptive activity remained constant over time and was not influenced by calcium content of the diet or reproduction. Serum concentrations of the bone deposition marker, ALP, differed significantly between diets for reproductive females over the reproductive bout, and between reproductive females and non-reproductive mice consuming the low-calcium diet. These differences correspond to observed differences in BMD, thus it appears that the observed reduction in BMD in reproductive females can be attributed to a change in bone formation, rather than resorptive activity. Decreases in serum ALP has been associated with a reduction in BMD, however this result has not been consistently demonstrated, suggesting that mineral mobilization strategies may differ between species. For example, no difference was found in serum ALP concentrations, or other markers of bone formation and resorption (osteocalcin and c-telopeptide type I collagen, respectively), between rats fed either low or adequate calcium diet for 10 weeks, although low calcium group exhibited a significant decrease in BMD in the vertebrae, femur and tibia (Kim et al., 2009). ALP has also been shown to be elevated in some cases in response to calcium

availability in the diet. In rabbits consuming a low calcium diet, serum ALP was significantly elevated and BMD of whole body, lumbar spine, femur and tibia was significantly lower relative to rabbit consuming a control diet (Mehrotra et al., 2006). Plasma ALP is elevated in human patients with osteomalacia relative to patients with no bone diseases (Peach et al., 1982), and a homogenate of rat bone cells and extracellular matrix cultured in low calcium medium exhibited significantly greater ALP activity than cells cultured in control medium (Yoshimura et al., 1996). Contrasting the fluctuations in bone formative activity associated with reproduction and a low-calcium diet against the lack of change in bone resorptive activity suggests that bone loss in white-footed mice can be attributed to a decrease in formative activity relative resorptive activity.

The extent of bone loss experienced by reproductive females on the low-calcium diet intimates that calcium investment in reproductive effort is prioritized over maintenance of the maternal skeleton. The extent that BMD was reduced in reproducing females consuming a low-calcium diet would likely increase fracture risk and may represent an exhausted calcium reserve that could not be efficiently replenished given the amount of calcium in the diet. This is evidenced by the condition of the females at weaning; all reproductive females on the low calcium diet displayed appendicular skeletal deformation and a resistance to placing weight on their limbs during locomotion (CMBS, personal observation). In an environment where calcium availability fluctuates or is abundant, it is possible that bone loss associated with offspring production does not yield long-term consequences. Our results show evidence that bone loss is reversible after weaning when sufficient dietary calcium is available, as has reported previously in other species (eg. Bezerra et al., 2004; Kovacs, 2005; Miller and Bowman, 2004). However, female white-footed mice undergo postpartum estrous, which could feasibly reduce or eliminate

the window of time available for recuperating from bone loss associated with previous reproduction before initiating a subsequent pregnancy. As such, the effects of reduced calcium intake would likely be more pronounced during subsequent reproductive bouts.

This relationship could parallel observed effects of reproduction on maternal survival in red deer (*Cervus elaphus*), in which the production of offspring reduces both a mother's probability of surviving to the next reproductive bout and the probability of subsequent offspring production (Clutton-Brock et al., 1982). The reduction in survivorship and future reproduction was suggested to be attributed to a depletion of fat stores, which had been mobilized to support current reproductive efforts (Clutton-Brock et al., 1982).

Our study has addressed fecundity, one of the variables that determines individual fitness, and how it is influenced by an interaction of endogenous and exogenous calcium availability. Fecundity drives lifetime reproductive output, and in this case has shown a marked response to calcium availability and potential physiological costs associated with providing sufficient calcium to developing offspring. The next step towards understanding this overall relationship is to take into account resource investment in individual young to determine if quality is compromised when calcium is scarce. As successful skeletal mineralization is essential for offspring survival, we predict that calcium investment in offspring will be prioritized over maternal skeletal condition when dietary calcium is limited.

We have demonstrated how calcium can affect reproductive performance and maternal condition in a manner that has been previously described within an energetic or theoretical context. Given the important role that calcium plays in offspring production, we argue that monitoring calcium availability and allocation may provide a robust framework with which to test life-history trade-offs and reproductive strategies in vertebrates.

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Table 1. Composition and content of custom manufactured diets.

	Low-Calcium	Standard
Composition		
Energy (Kcal/g)	3.8	3.7
Protein (% kcal from)	18.7	18.9
Carbohydrate (% kcal from)	66.6	66.2
Fat (% kcal from)	14.7	14.9
Calcium (% weight)	0.02	0.6
Phosphorus (% weight)	0.4	0.4
Vitamin D (IU/Kg)	2200	2200
Ingredients		
Casein (g/Kg)	200.0	200.0
L-Cystine (g/Kg)	3.0	3.0
Sucrose (g/Kg)	336.758	336.758
Corn Starch (g/Kg)	270.0	255.6
Maltodextrin (g/Kg)	50.0	50.0
Soybean Oil (g/Kg)	60.0	60.0
Cellulose (g/Kg)	40.0	40.0
Mineral Mix, Ca-P Deficient 79055 (g/Kg)	18.0	18.0
Potassium Phosphate, monobasic (g/Kg)	11.43	11.43
Calcium Carbonate (g/Kg)	0.4	14.9
Ferric Citrate (g/Kg)	0.3	0.3
Vitamin Mix, Teklad 40060 (g/Kg)	10.0	10.0
Ethoxyquin, antioxidant (g/Kg)	0.012	0.012

Table 2. Summary of comparisons within reproductive females or dietary treatments of bone mineral density (BMD) of femur, tibia, and lumbar vertebrae (L4-L6; Lumbar), and concentrations of alkaline phosphatase (bone formation marker) and pyridinoline crosslinks (bone resorption marker).

Group	Variables	Femur BMD	Tibia BMD	Lumbar BMD	Formation marker	Resorption marker
Reproductive females	Diet x Time	~	*	*	ns	ns
	Diet	-	-	-	**	ns
	Time	-	-	-	ns	ns
Low-Ca diet	Reproductive status x Time	ns	ns	ns	ns	ns
	Reproductive status	*	*	*	*	ns
	Time	~	*	***	ns	ns
Standard diet	Reproductive status x Time	ns	ns	ns	ns	ns
	Reproductive status	*	~	~	ns	ns
	Time	*	***	**	ns	ns

ns – not significant
- not tested
~ $P = 0.05$ - 0.058
* $P < 0.05$
** $P < 0.001$
*** $P \leq 0.000$

Fig. 1 Relationship between individual daily food intake and reproductive week for female white-footed mice consuming a low-calcium or standard diet. Weeks 1-3 correspond with period of gestation and weeks 4-6 correspond with period of lactation. Intake for non-reproductive mice consuming the standard diet is shown with a dotted line for comparison.

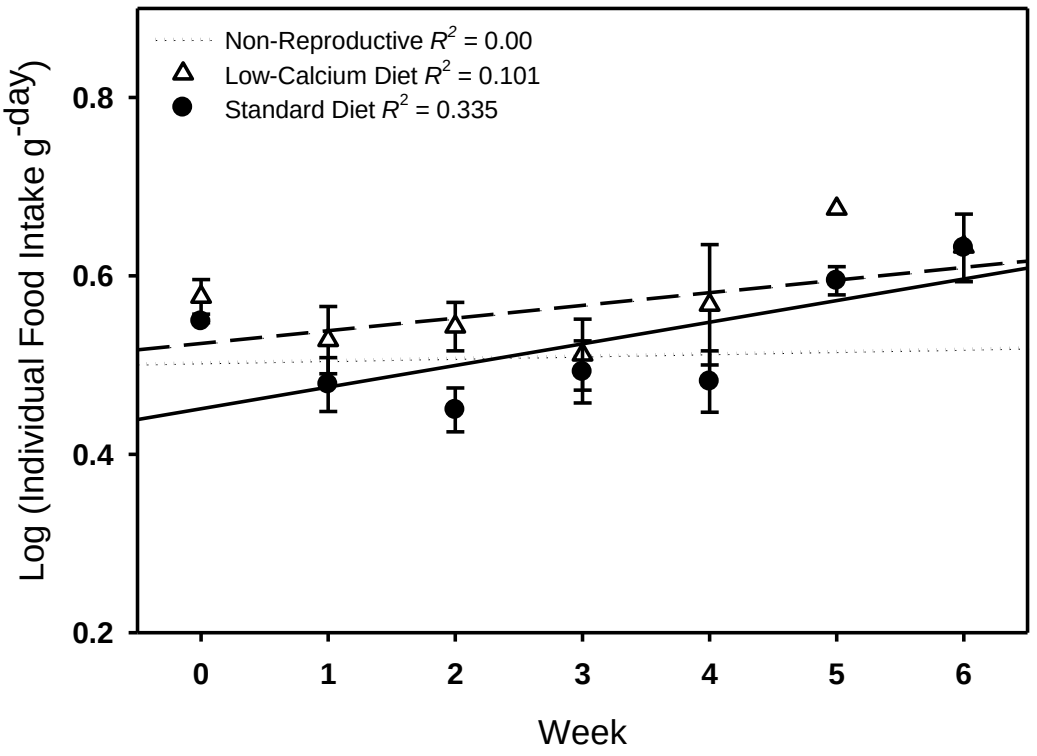
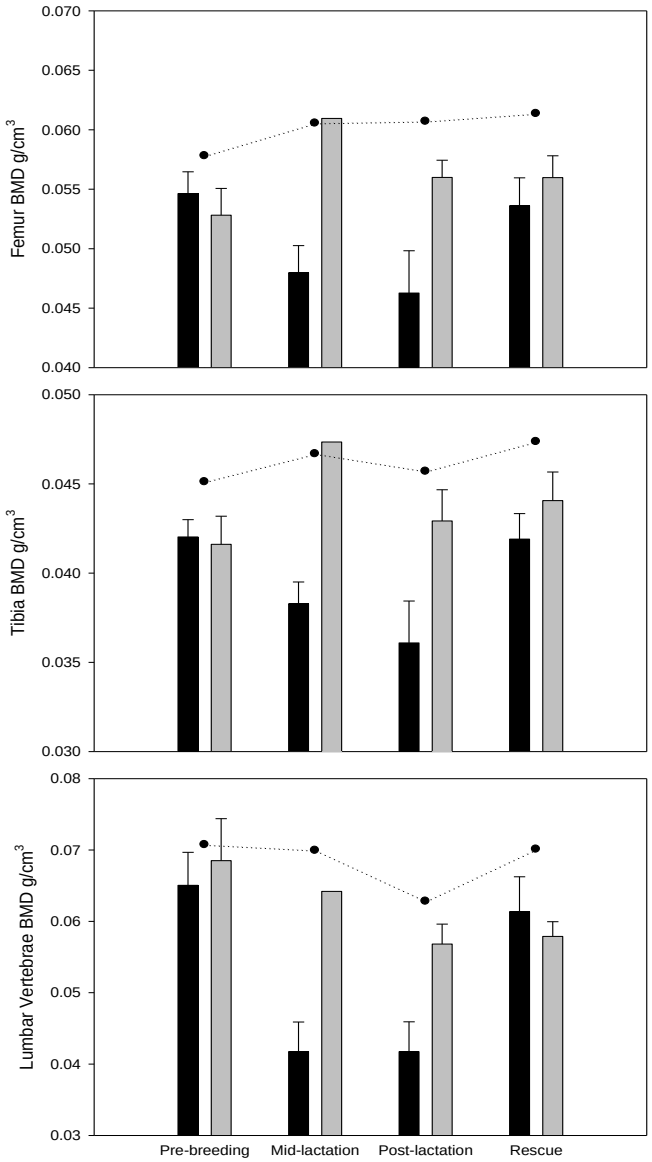


Fig. 2 Bone mineral density (BMD) of femur (A), tibia (B) and lumbar vertebrae (L4-L6; C) for reproductive and non-reproductive female white-footed mice on a low-calcium or standard diet at four sampling period. Pre-breeding = 2 days prior to breeding, Mid-lactation = 15-23 days post-partum, Post-lactation > 30 days post-partum, Rescue = 25 days after all mice were returned to the standard diet. Mean BMD for non-reproductive mice consuming the standard diet is shown with a dotted line for comparison.



II. Patterns of calcium and mineral investment into offspring production in response to calcium intake

Abstract

The production of offspring requires a substantial resource investment from the parent, which can be derived from both exogenous and endogenous sources. In mammals, females are exclusively responsible for the transfer of resources during gestation and lactation. The growth and mineralization of offspring bones constitutes a significant calcium demand on the mother, which often cannot be met by augmenting dietary calcium intake alone. Female mammals frequently mobilize calcium from their own skeleton in attempt to meet this demand, and, since the mobilization of skeletal calcium is inversely related to maternal calcium intake, it is generally assumed that bone mobilization compensates for dietary deficiencies. The potential cost associated with bone mobilization presumably limits the amount of endogenous calcium that can be transferred to developing young, therefore females may adjust the quantity of mineral allocated to reproductive efforts by either reducing the number of young produced, or by reducing that amount of mineral that is allocated to individual offspring. We provided reproducing female white-footed mice (*Peromyscus leucopus*) with either a low-calcium or standard diet to test if calcium available in the maternal diet affected reproductive output or mineral investment in offspring. There was no difference in the number of pups produced between diets, but pups produced by females on the low-calcium diet had significantly reduced femur widths and humerus ash concentration, and both femurs and humeri contained

significantly less mineral overall. Pups from females on the low-calcium diet also had lower body concentrations of calcium and lower calcium and phosphorous content in both the femur and humerus. Although previously shown that white-footed mice increase mobilization of bone mineral when calcium intake is low, our results suggest that bone mobilization did not completely compensate for a reduction in calcium intake, and that females reduced the quantity of mineral allocated per offspring instead of reducing the number of offspring produced.

Introduction

The investment in offspring production relative resource availability is one of the main concepts behind many models of reproductive trade-offs and life histories. The materials that a parent allocates to the production of offspring are derived from a resource pool that can be limited extrinsically or intrinsically. Extrinsic limitations are most commonly represented by the limited availability of food or some specific dietary component, whereas investment of resources into reproduction may be intrinsically limited by a variety of factors, such as a parent's capacity to absorb ingested nutrients or mobilize somatic stores of energy and nutrients. To maximize the likelihood of offspring survival, Smith and Fretwell proposed that the amount of resources invested in an individual offspring will be inversely proportional to the number of offspring that the parent can produce (Smith and Fretwell, 1974). In this case, investment in offspring is generally quantified as individual offspring mass and resources available for investment are assumed to be constant (Charnov and Ernest, 2006). Following this model, a reduction in resource availability should result in a proportional reduction of resources invested in offspring production, manifested as a reduction in resources allocated to individual offspring or a reduction in number of offspring produced.

In addition to a considerable demand for both energy and protein, the production of vertebrate offspring requires a substantial mineral investment for the mother. This mineral, mostly calcium, is predominantly allocated to the mineralization of fetal and neonate bone. In mammals, mothers transfer mineral to their progeny via the placenta during gestation, and via milk postpartum. The elevated demand for calcium during this time period usually requires mothers to increase their resource pool, which can be accomplished by elevating exogenous mineral intake, by mobilizing endogenous resources, i.e. mineral stored in their bones, or by some combination of the two. Several species exhibit elevated bone resorptive activity as a result of a decrease of dietary calcium intake (eg. Boelter and Greenberg, 1943; Brommage and DeLuca, 1985; Peng et al., 1988), illustrating both the importance of providing sufficient calcium to developing young and the interrelatedness between endogenous and exogenous calcium sources.

The mobilization of mineral from the maternal skeleton and consequent maternal bone loss can be viewed as a physiological cost of reproduction (Speakman, 2008). As bone mineral is lost, the likelihood of sustaining a fracture increases and maternal skeletal condition is therefore compromised (Broe et al., 2000; Reilly and Burstein, 1975). As such, bone mineral can presumably only be mobilized to a certain extent before the costs incurred by this activity are no longer offset by the benefit of successful reproduction. Thus, the amount of calcium that is available to invest in offspring production is limited both by intrinsic and extrinsic factors. Since diet plays a role in the degree to which bone is resorbed, exogenous calcium availability may influence this reproductive trade-off, and mothers may respond to limited calcium intake by curtailing investment in offspring production.

The effects of maternal diet on offspring bone characteristics have been studied most frequently in humans. It has been shown, for example, that women with generally low calcium intake responded to calcium supplementation by increasing fetal bone mineralization (Koo et al., 1999), and the intake of fat, milk and magnesium by women during late pregnancy is a strong predictor of their offspring's bone mineral density at 16 years old (Yin et al., 2010). However, since humans usually produce one progeny per birth, mothers cannot adjust litter size relative to mineral availability. Guinea pigs that were fed a vitamin D deficient diet (which can impair calcium absorption) during gestation have also been shown to produce offspring with diminished bone mineral content, and, in this case, there was no concurrent reduction in litter size (Finch et al., 2010). However, the effects of calcium intake during gestation and lactation on the investment of calcium and other minerals to offspring production have yet to be tested in small mammals. Given the considerable amount of calcium required to produce young in addition to a limit to the amount of mineral that can be mobilized from maternal bone, a reduction in calcium available to the mother should result in a reduction of calcium invested in offspring production. Following the Smith-Fretwell model, mothers should either reduce the amount of calcium allocated to individual offspring, or limit the number of offspring produced, thus maintaining the amount of calcium allocated to an individual offspring. We tested this by manipulating calcium intake of white-footed mice (*Peromyscus leucopus*) and assessing how calcium, and mineral in general, was allocated to offspring in response to maternal intake relative to reproductive output.

Deer mice (*P. maniculatus bairdii*), a closely related species, show a strong inverse relationship between litter size and individual offspring mass at birth and at weaning, (Myers and Master, 1983), although Fleming and Rauscher (Fleming and Rauscher, 1978) found no relationship between litter size and individual offspring size in white-footed mice. Litter size of

both species was correlated with maternal mass (Morris, 1986; Myers and Master, 1983), and maternal mass was also positively correlated to individual offspring mass at birth and at weaning in deer mice (Myers and Master, 1983), suggesting that the investment of resources into offspring production is influenced by maternal condition (e.g. the amount of energy and nutrients stored in maternal tissues). If female white-footed mice experience a reduction in exogenous calcium availability, we expect them to adjust investment of calcium and other mineral into offspring production as a means of maintaining their own condition, by either decreasing the amount of calcium and overall mineral invested per offspring, or by reducing the number of offspring produced in a litter.

Materials and Methods

We maintained 15 female and 8 male white-footed mice obtained from a captive-bred population (Peromyscus Genetic Stock Center, University of South Carolina, Columbia, SC, USA) at an animal research facility at Auburn University (Auburn, AL, USA). Females were housed in pairs in 29 x 19 x 12.5 cm polypropylene rodent enclosures (Lab Products, Inc., Seaford, DE USA), 3-4 males were housed in larger polypropylene containers, and all were maintained at 25C on a 16:8 light cycle. Over the course of 50 weeks, we provided each female with 4 breeding opportunities by placing a randomly selected male in a female container for 14 days. Females were never paired with the same male twice. We gave females *ad libitum* access to one of 2 custom manufactured diets that varied only in calcium content (Table 1; Harlan Teklad, Madison, WI, USA). We assigned 7 females to a low-calcium diet (0.02% dry mass) and 8 females to a standard diet (0.6% dry mass), which contained the recommended amount of calcium for reproducing mice (Harlan Teklad, pers. comm.).

We measured weekly food consumption of females by calculating the difference between the mass of food added to the container and the mass of food remaining after 7 days. Females were maintained as pairs for the first 2 weeks of gestation, and were separated into separate boxes after that in order to monitor reproductive output of each individual. Therefore, individual food intake was only measured from week 3 onward. From these data, we estimated calcium intake over the course of late gestation and lactation. Females were monitored daily in order to assess parturition date, and pups were weighed and counted 7 days after that. We also measured tail length at these times in order to assess size of individual pups. We continued to measure and weigh pups every seven days until they were completely weaned (28 days). We euthanized 48 out of a total of 77 pups produced over this time period at 28 days for the following composition analyses.

Bone morphology and composition

We excised the left femur and humerus from pup carcasses and cleaned them first by manually removing tissue and then by placing bones in an ultrasonic cleaner for 30 minutes (Sper Scientific Ltd., Scottsdale, AZ, USA). Several bones sustained fractures during the cleaning process; bones that remained intact were photographed on a grid using a digital camera attached to a dissecting microscope. From the photographs, we measured the length of the bone shaft and the width of the bone mid-shaft using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA). We then placed the bones in a soxhlet apparatus for 12h, using a 2:1 mixture of PET ether and acetone, to remove any lipids and then dried them overnight in an oven at 100C (Binder drying oven FED 115-UL, Binder Inc., Great River, NY, USA) in order to obtain fat free dry mass for the individual bones. We then ashed bones in a muffle furnace

(Isotemp Muffle Furnace, Fisher Scientific, Dubuque, IA, USA) at 500C for 12 hours, and remaining ash was weighed to establish percent mineral of fat free dry bone mass. We then digested ash in trace mineral grade nitric acid on a heating block at 100C for one hour, and diluted digested sample with nanopure water. Content of calcium, magnesium, phosphorous and iron (Ca, Mg, P, Fe) was then measured by inductively-coupled plasma optical emission spectrometry (ICP; Perkin Elmer Optima 7300DV, Waltham, MA, USA). We added an internal silver (Ag) standard to each sample to quantify recovery of minerals, and analyzed each sample in duplicate. Mineral concentrations were averaged across duplicates, and then used to calculate total mass of each mineral present in each bone.

Whole body composition

After excising the femur and humerus, we dried carcasses at 100C for 12h and then homogenized each sample. We then used 2 subsamples (0.276 ± 0.0014 g) of the homogenate which either underwent drying and fat extraction as described above, or were ashed, digested and analyzed via ICP as described above.

Statistical analysis

We compared the number of offspring produced per litter over the course of the 4 breeding opportunities between diets using ANOVA. When females produced more than one litter, a female's litter size was based on the average of all litters produced. We used general linear models (PROC GLM) to test the relationships between litter size and mineral content of pups and pup bone size, maternal food intake over time (from about the 3rd week of pregnancy until the 3rd week of lactation), and whether maternal calcium intake interacted with these

relationships. We also used GLM to assess if mean maternal food intake over the course of gestation and lactation was related to mineral content of pups and bone size measurements. We used a two level nested ANOVA, with pup nested within mother, to compare whole body and bone mineral composition of pups, mass of pups at days 7, 21 and 28, tail length of pups at days 7, 21 and 28 and pup bone size and mass between calcium content of mothers' diet (PROC GLM). All analyses were performed in SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

There was no significant difference in total number of offspring produced by mothers on the low-calcium or standard diet ($F_{1,13} = 1.30$ $P = 0.216$) for the pups that were used for composition analyses. There was also no difference in litter sizes produced throughout the study between the dietary groups ($F_{1,15} = 0.40$, $P = 0.535$). There was a significant difference in food intake between mothers on the low-calcium and standard diet ($F_{1,57} = 4.49$, $P = 0.0396$) and over time ($F_{6,57} = 13.33$, $P < 0.0001$), but these two variables did not interact to affect maternal intake ($F_{4,57} = 2.14$, $P = 0.0913$; Fig. 1). There was no significant difference between diets for pup mass or tail length at day 7, 21 or 28 ($F_{1,49} \leq 1.10$, $P \geq 0.299$ in all models; Table 2). Litter size had no significant effect on mean percent ash of pup body, femur or humerus, ash mass of femur or humerus, or femur and humerus length and width, produced by individual females, nor did it interact with maternal diet to affect these variables ($F \leq 3.61$, $P \geq 0.124$ in all models).

Bone morphology and composition

Pups produced by mothers on the standard diet had significantly wider femurs at mid-shaft than those from mothers on the low-calcium diet (Table 3). There were no other differences in bone length of pups between diets (Table 3).

Mothers that consumed the standard diet produced pups with significantly higher percentage of ash in the humerus than mothers that consumed the low-calcium diet (Fig. 2, Table 4), and total ash content was higher in both the humerus and the femur (Fig. 2, Table 4).

Percent Ca and P did not vary between diets for the femur or humerus (Tables 5 - 6) although both bones exhibited a higher concentration of Na and Mg for pups in the low-calcium group (Tables 5 - 6). Ca and P content were significantly greater in the femur, and, although non-significant, was higher in the humerus as well, for pups from mothers that consumed the standard diet (Tables 5 - 6). There was no difference between diets for Na or Mg content in either bone (Tables 5 - 6).

Whole body composition

There was no significant difference in percent ash content of whole bodies of pups from mothers on either diet (Fig. 2, Table 4).Pups produced by mothers on the low-calcium diet exhibited significantly lower body concentrations of calcium than those from mothers in the standard diet group (Table 7). Phosphorus, magnesium, sodium and iron concentrations in the body did not vary between diets (Table 7).

Discussion

Our results show that maternal calcium intake influenced calcium and overall mineral allocation to individual offspring, but did not affect reproductive output, illustrating the influence of exogenous calcium availability on the degree of mineral investment into offspring production. The amount of calcium that mothers consumed was positively related to the overall composition of pups with regard to calcium concentration.

The morphology and composition of long bones was also influenced by maternal diet, however the response differed between specific bones. We found no difference in bone length between diets for either the femur or the humerus of pups, however pups produced by mothers consuming a low-calcium diet exhibited a reduction in width was not found in the humerus. Total mineral mass deposited in both bones was significantly lower in pups in the low-calcium group. Pups from the standard group had, on average, 60-70% more calcium in their bones. Phosphorus content mirrored calcium content in both bones, illustrating the tight correlation between this element and calcium in bone, and suggesting that calcium availability limits phosphorous accretion. Proportion of ash in humeri was diminished in low-calcium group, suggesting an overall reduction in mineralization that was not observed in femurs. Although the relative composition of both sodium and magnesium was greater for the low-calcium group, there was no difference in the total mass of either of these elements in either bone. Therefore differences in ash concentration can be attributed to variations in calcium content. .

The variation in response to maternal calcium intake between the femur and humerus of offspring may reflect a general difference in ossification rates of each bone (e.g. Chahoud and Paumgartten, 2005). Ossification rate may correspond to degree of mechanical strain than a specific bone may sustain (Farnum et al., 2008; Lerner et al., 1998). White-footed mice are

partially arboreal (Graves et al., 1988), and this mode of locomotion may place relatively greater stress on the hind limbs. Thus, the pattern of mineral accretion observed in this study indicates it may be relatively more important for the femur to resist mechanical strain than the humerus early in life.

There was no difference in litter size or mass of individual offspring, and we found no relationship between litter size or any of the pup bone and mineral characteristics. Mothers respond to a calcium deficient diet by limiting the amount of calcium allocated to individual young rather than by limiting reproductive output. White-footed mice were not proportionally reducing investment in offspring production as predicted in the Smith-Fretwell model in which mothers reduce reproductive output relative to a reduction in resources available for allocation to young (Smith and Fretwell, 1974).

In white-footed mice, the extent that mineral is mobilized from bone, characterized by diminished bone mineral density, is significantly greater for females that were consuming a low-calcium diet (Chapter I). This relationship has been frequently observed in other mammal species and suggests that bone mobilization serves to compensate for inadequate intake of dietary calcium. However, mothers in this study that consumed a low-calcium diet also allocated less calcium to individual progeny, suggesting that there is a limit to the amount of mineral that can be mobilized from bone, which, in periods of extreme dietary calcium deficiency may be insufficient to meet offspring requirements.

The limitations that the availability of endogenous resources impose on reproductive investment have been best described within the context of adipose stores. Body fat, i.e. stored energy, has been linked to reproductive performance in a variety of species, including squamate reptiles, daphnia, lagomorphs and ungulates (Doughty and Shine, 1997; Fortun-Lamothe, 2006;

Parker et al., 2009; Valencak et al., 2009). While rodents and other small mammals rely more heavily on exogenous energy acquired during the reproductive process (i.e. an income breeding strategy), their dependence on stored nutrients (i.e. capital) such as calcium may vary in response to dietary availability. Under adequate dietary conditions, 19% of calcium in milk produced by rats is derived from the maternal skeleton (Brommage, 1989). As calcium intake decreases, lactating rats increase the amount of bone that is mobilized and subsequently lost (Brommage and DeLuca, 1985). Thus, in environments where dietary calcium may not be consistently available, the capacity to respond to fluctuations is advantageous for maximizing reproductive success in vertebrates such as white-footed mice.

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Table 1. Composition and content of custom manufactured diets.

	Low-Calcium	Standard
Composition		
Energy (Kcal/g)	3.8	3.7
Protein (% kcal from)	18.7	19.1
Carbohydrate (% kcal from)	66.5	65.9
Fat (% kcal from)	14.8	15.0
Calcium (% weight)	0.1	0.85
Phosphorus (% weight)	0.4	0.4
Vitamin D (IU/Kg)	2200	2200
Ingredients		
Casein (g/Kg)	200.0	200.0
L-Cystine (g/Kg)	3.0	3.0
Sucrose (g/Kg)	334.658	330.458
Corn Starch (g/Kg)	270.0	255.6
Maltodextrin (g/Kg)	50.0	50.0
Soybean Oil (g/Kg)	60.0	60.0
Cellulose (g/Kg)	40.0	40.0
Mineral Mix, Ca-P Deficient 79055 (g/Kg)	18.0	18.0
Potassium Phosphate, monobasic (g/Kg)	11.43	11.43
Calcium Carbonate (g/Kg)	2.5	21.2
Ferric Citrate (g/Kg)	0.3	0.3
Vitamin Mix, Teklad 40060 (g/Kg)	10.0	10.0
Ethoxyquin, antioxidant (g/Kg)	0.012	0.012

Table 2. Individual pup mass and tail length at day 7, 21 and 28, post-parturition of pups produced by mothers that consumed either a low-calcium (Low) or standard (Std) diet. Means are shown ±SE

	Day 7				**Day 21**				**Day 28**			
	$\bar{X}$ **Low**	$\bar{X}$ **Std**	$F_{1,18}$	***P***	$\bar{X}$ **Low**	$\bar{X}$ **Std**	$F_{1,18}$	***P***	$\bar{X}$ **Low**	$\bar{X}$ **Std**	$F_{1,18}$	***P***
Mass, g	5.19 ± 1.01	4.80 ± 0.68	0.11	0.745	10.10 ± 1.14	8.97± 0.64	0.89	0.360	12.06 ± 0.72	11.55 ± 0.76	0.19	0.665
Tail length, mm	20.09 ± 1.13	18.48 ± 1.27	0.72	0.407	46.43 ± 5.10	42.36 ± 4.39	0.34	0.566	52.53 ± 5.81	47.62 ± 5.08	0.37	0.548

Table 3. Measurements of shaft length and width of femurs and humeri from pups produced by mothers that consumed either a low-calcium (Low) or standard diet.

	$\bar{X}$ Low ± SE	$\bar{X}$ Standard ± SE	F	df	*P*
Femur shaft length, mm	10.620 ± 0.162	10.840 ± 0.173	0.69	1, 25	0.415
Femur mid-shaft width, mm	1.036 ± 0.0320	1.162 ± 0.0300	7.02	1, 25	0.015
Humerus shaft length, mm	8.379 ± 0.221	8.184 ± 0.0708	0.68	1, 24	0.422
Humerus mid-shaft width, mm	0.850 ± 0.0418	0.866 ± 0.0195	0.26	1, 24	0.617

Table 4. Percent ash, based on a fat-free dry mass (ffdm) basis, of whole body, femur and humerus as well as total mineral mass of femur and humerus of pups produced by mothers that consumed either a low-calcium (Low) or standard diet.

	$\bar{X}$ Low ± SE	$\bar{X}$ Standard ± SE	F	df	*P*
% ash body (ffdm)	8.816 ± 0.241	8.936 ± 0.242	1.40	1, 27	0.250
% ash femur (ffdm)	44.507 ± 2.111	47.089 ± 2.224	0.57	1, 16	0.461
% ash humerus (ffdm)	31.250 ± 6.0649	51.358 ± 0.859	9.20	1, 16	0.009
Femur ash, mg	3.695 ± 0.288	6.734 ± 0.572	13.96	1, 16	0.002
Humerus ash, mg	2.381 ± 0.194	4.204 ± 0.332	23.80	1, 16	0.0002

Table 5. Total and percent Calcium (Ca), phosphorous (P), magnesium (Mg) and sodium (Na) content present in the femur of pups produced by mothers that consumed either a low-calcium (Low) or standard diet. % recovered represents mean percent of each element detected via ICP relative to concentration of a known standard. Proportion of mineral presented on a fat-free dry mass basis.

Element	***% Recovered***	$\bar{X}$ **Low ± SE**	$\bar{X}$ **Standard ± SE**	$\mathbf{F_{1,13}}$	***P***
Ca mg	100.3 ± 5.4	3.80 ± 0.22	6.25 ± 0.43	12.15	0.005
%		43.2 ± 1.22	44.1 ± 0.92	0.31	0.589
P mg	101.6 ± 4.0	3.07 ± 0.25	4.85 ± 0.32	14.15	0.003
%		35.9 ± 1.20	32.4 ± 3.25	0.52	0.482
Mg mg	101.5 ± 2.2	0.157 ± 0.0082	0.177 ± 0.013	0.83	0.382
%		1.68 ± 0.10	1.32 ± 0.11	4.26	0.060
Na mg	92.7 ± 4.0	0.656 ± 0.037	0.620 ± 0.085	0.08	0.776
%		7.77 ± 0.35	4.66 ± 0.72	7.88	0.014

Table 6. Total and percent Calcium (Ca), phosphorous (P), magnesium (Mg) and sodium (Na) content present in the humerus of pups produced by mothers that consumed either a low-calcium (Low) or standard diet. % recovered represents mean percent of each element detected via ICP relative to concentration of a known standard. Proportion of mineral presented on a fat-free dry mass basis.

Element	***% Recovered***	$\bar{X}$ **Low ± SE**	$\bar{X}$ **Standard ± SE**	$F_{1,10}$	***P***
Ca mg	100.3 ± 5.4	2.76 ± 0.23	3.80 ± 0.37	4.18	0.075
%		45.7 ± 1.37	43.1 ± 0.69	2.40	0.150
P mg	101.6 ± 4.0	2.25 ± 0.23	3.06 ± 0.28	4.80	0.056
%		37.8 ± 1.39	34.9 ± 0.46	3.46	0.090
Mg mg	101.5 ± 2.2	0.104 ± 0.022	0.102 ± 0.022	0.01	0.923
%		1.87 ± 0.23	1.14 ± 0.21	5.26	0.043
Na mg	92.7 ± 4.0	0.624 ± 0.045	0.563 ± 0.0026	1.59	0.235
%		12.0 ± 0.99	6.88 ± 0.61	19.15	0.001

Table 7. Percent Calcium (Ca), phosphorous (P), magnesium (Mg), sodium (Na) and iron (Fe) content present in the body of pups produced by mothers that consumed either a low-calcium (Low) or standard diet. % recovered represents mean percent of each element detected via ICP relative to concentration of a known standard. Proportion of mineral presented on a fat-free dry mass basis.

Element	**% Recovered**	$\bar{X}$ **Low ± SE**	$\bar{X}$ **Standard ± SE**	$F_{1,47}$	***P***
Ca %	95.2 ± 3.1	24.3 ± 0.60	27.4 ± 0.94	6.54	0.015
P %	108.2 ± 2.7	28.6 ± 0.25	28.7 ± 1.24	0.00	0.949
Mg %	99.5 ± 1.3	1.34 ± 0.027	1.30 ± 0.064	0.17	0.683
Na %	95.0 ±1.0	4.42 ± 0.11	3.87 ± 0.18	3.58	0.067
Fe %	101.9 ± 2.2	0.403 ± 0.022	0.367 ± 0.024	1.04	0.316

Fig. 1. Mean food intake (A) and calcium intake (B) from week 3 of gestation until weaning (week 7) by mothers consuming either a low-calcium or standard diet.

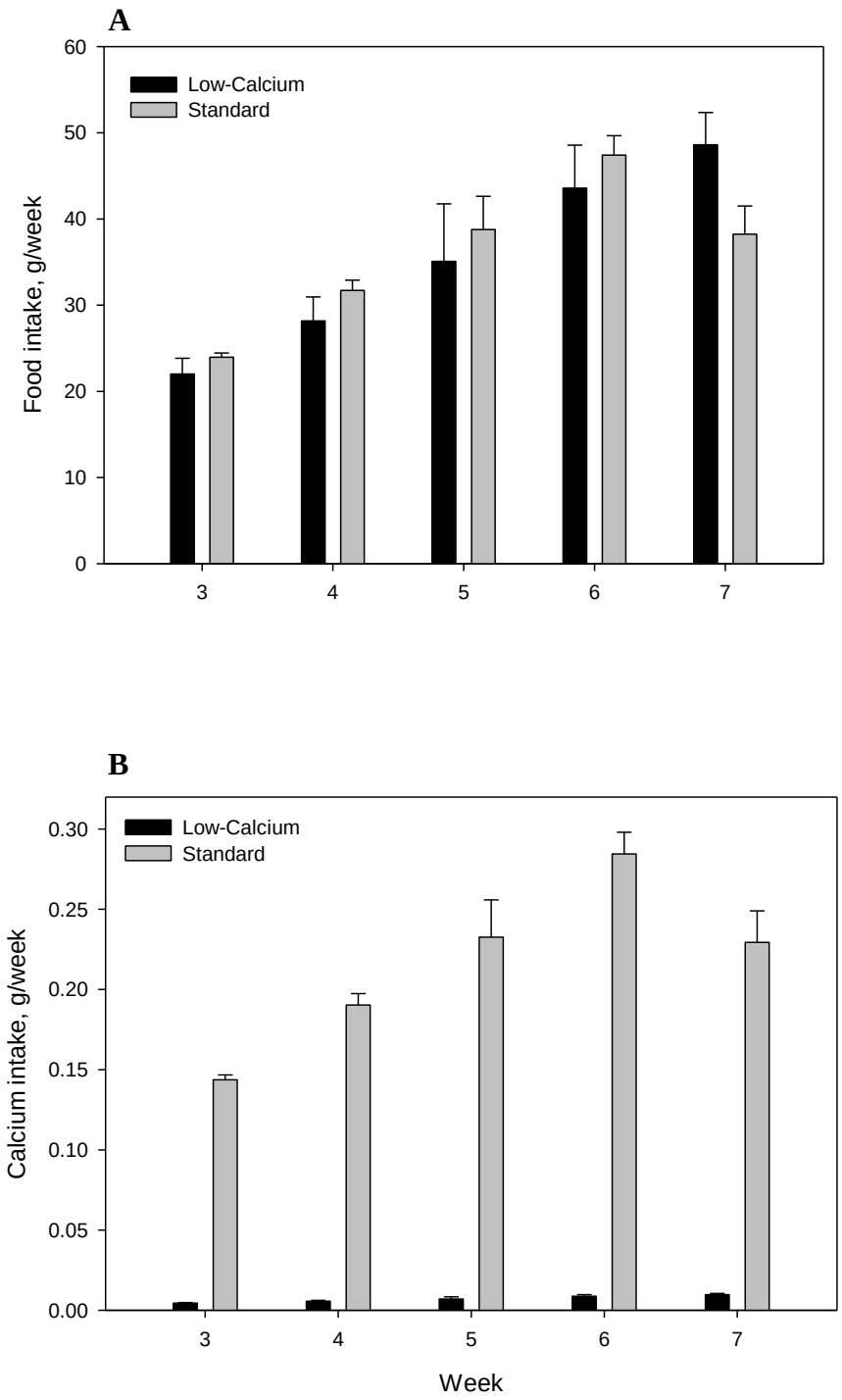


Fig. 2. Ash content of the humerus and femur of pups produced by mothers consuming either a low-calcium or standard diet.

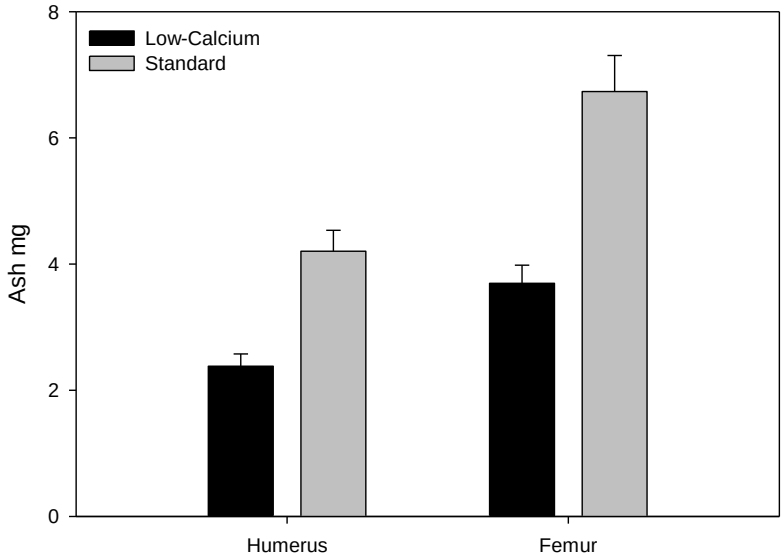


Fig. 3. Whole body ash composition of pups produced by mothers consuming either a low-calcium or standard diet. Values are presented on a fat-free dry mass (ffdm) basis.

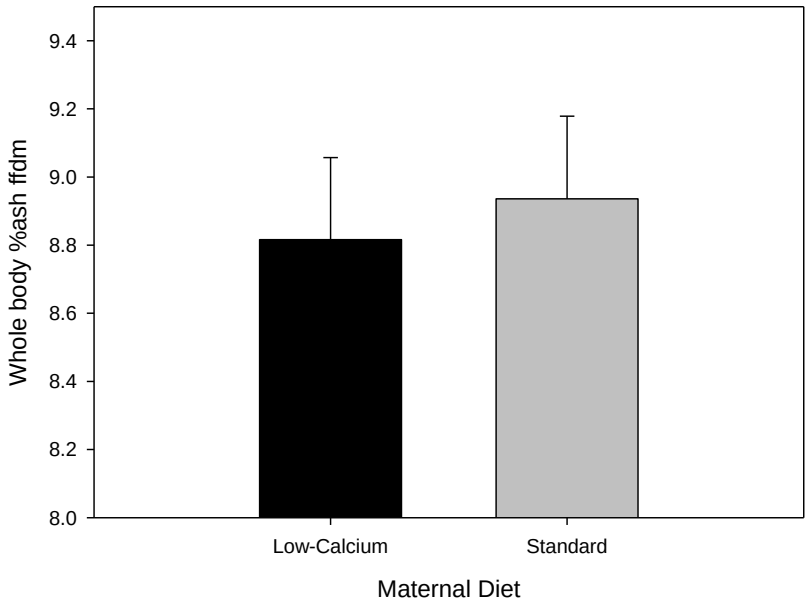
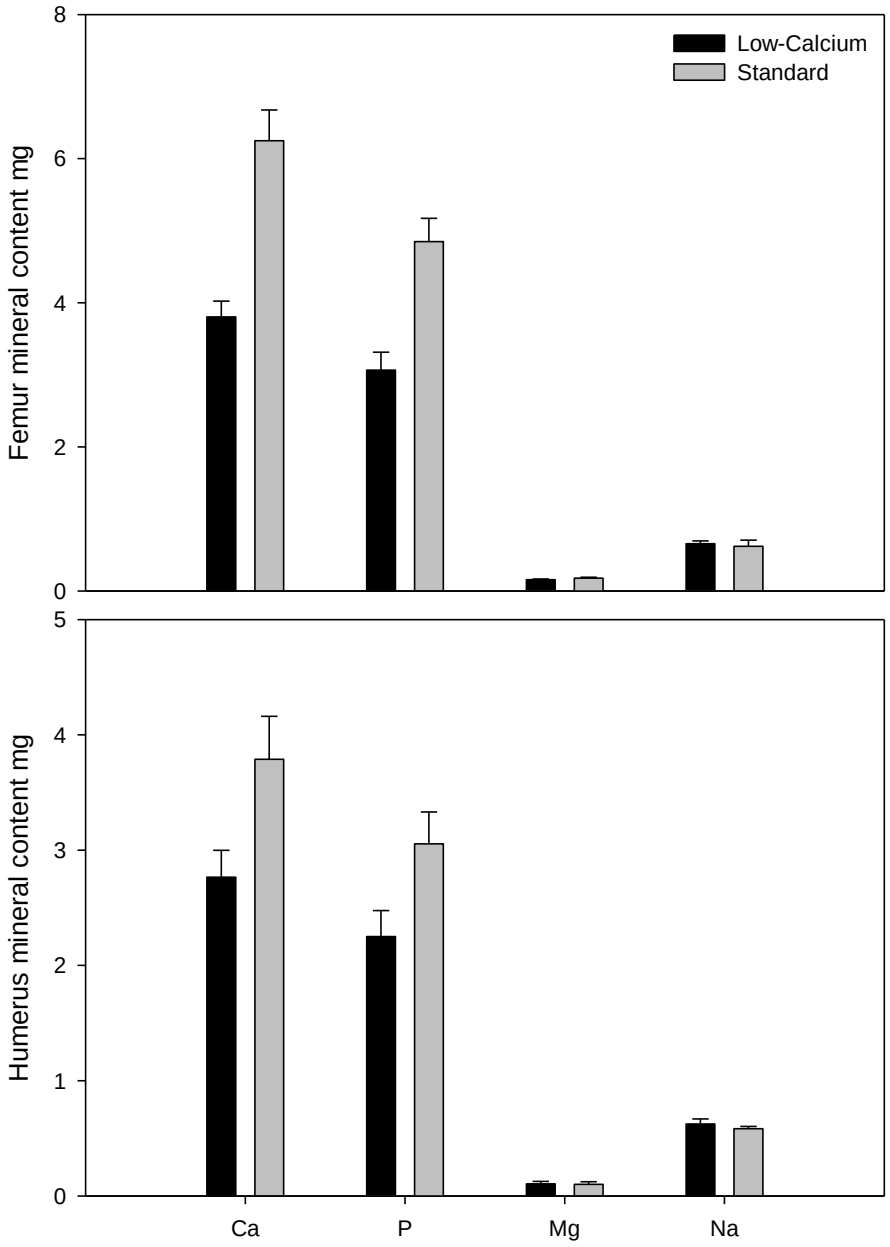


Fig. 4. Calcium (Ca), phosphorous (P), magnesium (Mg) and sodium (Na) content of the humerus and femur of pups produced by mothers consuming either a low-calcium or standard diet.



III. The effects of litter size and parity on bone metabolism during gestation and lactation

Abstract

During gestation and lactation, female mammals often mobilize endogenous nutrient reserves to support the resource demands of offspring production. Bone mineral is frequently resorbed from the maternal skeleton during this time to facilitate the rapid mineralization of offspring bones. The degree that bone is mobilized is influenced by dietary mineral intake, with bone mobilization appearing to compensate for any dietary mineral deficiencies. Presumably, there is a minimum amount of mineral that needs to be allocated to individual progeny for reproduction to be successful. As such, the production of more offspring should result in an increase in mineral demand during gestation and lactation. However, since there is also presumably a limit to the amount of bone loss that a mother can sustain, maternal characteristics may also limit bone metabolic activity. We investigated the relationship between reproductive output and bone metabolism in domestic sows (*Sus scrofa*) over the course of a reproductive bout. Additionally, we tested whether maternal mass and parity was related to bone metabolic activity. We measured serum concentrations of products of bone resorption and bone formation to assess changes in bone metabolic activity over the reproductive bout. Litter size significantly affected both resorptive and formative activity during lactation, but not during gestation. Litter mass affected bone formation during gestation, and both formation and resorption during lactation. Parity also affected bone formation during gestation and both formation and resoprtion

during lactation, with primiparous and relatively inexperienced females exhibiting higher concentrations of both products. This suggests that maternal bone mobilization is influenced by the number of young that are produced, and that mothers may be able to change their response to offspring demands with reproductive experience.

Introduction

The production of offspring requires parental resources be partitioned to the production of gametes and in many species, resource must also be supplied to support the growth and development of young post-conception. In mammals, post-conception care is primarily, if not exclusively, the responsibility of the mother. To support offspring development through gestation and lactation, mothers often rely both on increased intake of exogenous nutrients and the mobilization of their own somatic tissue stores. Allocating somatic resources to offspring reduces the pool of internal resources available for self-maintenance, and as a result maternal condition can be adversely affected. As a result, maternal investment iteroparous species is limited by a tradeoff between allocation to the current reproductive effort and maternal survival and future reproductive performance (Clutton-Brock, 1984; Lack, 1947, 1948; Pontier et al., 1993; Stearns, 1992; Williams, 1966).

The currency most often employed to illustrate this trade-off is energy, however the production of vertebrate offspring also requires, among other things, a sizable investment of minerals by the mother. In particular, there is a substantial demand for calcium to facilitate the ossification of fetal and neonate bones. Increasing mineral intake can help to support this demand; yet most mammals studied to date experience some degree of bone loss during reproduction which is attributable to the allocation of maternal bone mineral to offspring

development (e.g. Boelter and Greenberg, 1943; Kalkwarf and Specker, 2002; Lees et al., 1998; Liesegang et al., 2007; Prentice, 2000; Shahtaheri et al., 1999; Speakman, 2008; Zeni et al., 1999).

Both dietary and reproductive pressures likely affect changes in the rates of bone turnover that a female experiences during a reproductive event. Although the influence of diet on bone loss has been studied in several species of mammals, little attention has been given to the interactions between the loss of bone mineral by mother and size and number of offspring that she produces. In humans, women that gave birth to, and subsequently nursed, twins or triplets were shown to lose more bone than those that produced one offspring (Laskey et al. 1998). In rats, Peng et al. (1988) found that female rats that produced relatively large litters experienced greater bone loss than those that produced relatively fewer offspring (Peng et al., 1988). In contrast, Sengupta et al. (2005) found no difference in the bone mineral density of females rats that nursed either 2 or 6 pups (Sengupta et al., 2005).

Although the relationship between bone metabolic activity and reproductive output has received virtually no attention outside of a clinical context, there are clearly ecological ramifications. Presumably, there is a threshold at which the costs incurred on maternal skeletal condition are no longer offset by the benefit of producing offspring. Trading off maternal skeletal condition and reproduction would undoubtedly influence resource partitioning strategies as a means of maximizing lifetime reproductive output. In mice, there is a negative relationship between the mineral content of mothers' femurs at weaning and litter size which reaches a nadir for mothers rearing 13 pups (Hood, submitted), suggesting a limit to the amount of mineral that can be mobilized from bone during reproduction. Females rearing larger litters (i.e. 18 young)

had greater bone mineral at weaning attributable to a reduction in mineral allocation by mothers that cannibalized some of their young (Hood submitted).

Bone metabolic activity may also be intrinsically limited by other factors associated with maternal physiology, such as age or reproductive history, (Allali et al., 2007; Giesemann et al., 1998). There are numerous examples of age or number of prior reproductive bouts affecting reproductive output. For instance, young meerkats (*Suricata suricatta*) and lynx (*Lynx lynx*) produce fewer litters and smaller litter sizes than older individuals (Henriksen et al., 2005; Sharp and Clutton-Brock, 2010), and multiparous Columbian ground squirrels (*Spermophilus columbianus*) produced more successful young per litter than inexperienced females (Broussard et al., 2008). Multiparous guinea pigs (*Cavia porcellus*) are more efficient at converting energy to the production of offspring than primiparous females (Kunkele, 2000), and primiparous grey seals (*Halichoerus grypus*) exhibit significantly lower energy output during lactation than multiparous females (Lang et al., 2011). These age and parity related differences in reproductive performance are established by variation in the physiological efficiency of systems that support nutrients transfer to an across the placenta and into milk and/or an increase in the total amount of nutrients partition to these processes.

Trivers (1974) proposed that offspring use 'psychological weapons' to compete against parents for resources, however it is also likely that offspring also employ 'physiological weapons' both in utero and during lactation. In the case of calcium transfer, it has been shown that fetal mice can regulate placental transfer of calcium hormonally (reviewed in Kovacs, 2000) illustrating that mineral investment in offspring production in not determined exclusively by the mother. In a less direct manner, increased suckling activity by a large litter and/or larger young stimulates greater milk let down, and a byproduct of processes that maintain milk mineral

content – bone turnover will also be affected. Thus, we predict that if bone metabolism is driven by offspring demand, then a relationship should exist between reproductive output and bone metabolic activity which may, in part, be affected by maternal characteristics.

We used domestic sows (*Sus scrofa*) to investigate this relationship. Swine have undergone strong artificial selection for productivity; wild boars produce up to 5 young per litter (Servanty et al., 2007; Taylor et al., 1998) whereas the Yorkshire breed, which we used in this study, can produce more than 13 young per litter (Hoving et al., 2011). Wild boars exhibit a much higher natural mortality rate than other ungulates (Gaillard et al., 2000), which has been attributed to their relatively high reproductive output and associated energetic costs (Toigo et al., 2008). Thus, with even larger demands placed on domestic individuals, we anticipated that the costs of reproduction to be especially tractable in domestic sows.

It is unclear what the natural longevity of domestic sows is, as they are usually slaughtered at around 3 years of age. However, culling at 3 years is common practice associated with a decline in productivity or the presence of symptoms such as lameness (Anil et al., 2009), which may be indicative of skeletal pathologies. In a dietary manipulation study using reproducing swine, calcium and phosphorous content of the diet had no effect on maternal skeletal condition, however sows that produced larger litters (11-12 young) exhibited reduced metacarpal bending moments compared to those that produced smaller litters (6-7 young), as well as a decrease in rib and vertebra bone ash and femur thickness from first to second parity (Maxson and Mahan, 1986). Thus, sows are likely trading-off skeletal condition for reproduction, and may be doing so to a greater degree than their wild counterparts. Additionally, swine, in general, exhibit a greater reproductive effort, quantified by litter mass relative to maternal mass, relative to other mammals of similar size (based on Hood et al., 2011), which

would presumably make them more susceptible to physiological costs of reproduction. As with other mammals, primiparous and early parity sows tend to produce less offspring per birth (Edwards, 2002; Smith et al., 2008). Considering changes in reproductive output relative to reproductive experience within the context of bone mobilization and mineral allocation, it is feasible that efficiency of these functions may increase with parity in sows. As such, greater reproductive output should be associated with elevated bone metabolic activity, and reproductive experience should influence how mothers accommodate mineral demands of developing offspring.

Materials and Methods

We collected serum samples from 15 Yorkshire sows bred and maintained at the Auburn University Swine Teaching and Research Facility (Auburn, AL, USA). Sow age ranged from 10.5 – 50 months, and parity ranged from 0 to 6 prior births. All husbandry and feeding practices described followed standard protocols at the facility. All sows were bred with one male within 3 days of each other (9-11 October, 2009). Sows were placed on restricted feed throughout gestation, and since sows consumed all available food, there was no difference between individuals with regard to food intake. During gestation, sows were feed a diet contained 13% protein, 4.5% fat, 0.72% calcium, 0.06% phosphorous, and provided about 3,230 Kcal/kg metabolizable energy. During lactation, sows were provided food ad libitum, and feed a diet with 19% protein, 8.3% fat, 0.75% calcium, 0.63% phosphorus, and provided about 3,470 Kcal/kg metabolizable energy. Food intake during lactation was recorded from parturition to weaning for each sow. Sows were weighed prior to breeding and after giving birth, and number and mass of live-born young was recorded at birth. Sow frequently produce stillborn young, and since

mineral demands of lactation generally outweigh mineral transferred in utero, we only excluded data from sows that produced litters that contained less than 80% live-born young.

We drew approximately 1.0 mL of blood from a marginal ear vein, using a 22 gauge needle and a 3mL syringe, and immediately transferred it to 4mL glass serum collection tubes. Within 1h of collection, we centrifuged blood for 10 minutes at 3,000 rpm, drew serum off of the sample and stored it at -80C until analysis. We collected samples 8 times over the course of one reproductive bout: one day prior to breeding (day 1), day 42, 84 and 98 of gestation (each ± 5 days), the day of parturition, days 14 and 21 of lactation, and 3 days after weaning. We collected samples at approximately the same time of day (12:00 – 14:30) to control for diurnal fluctuations in the concentration of bone markers (Allen, 2003; Ladlow et al., 2002).

We measured serum concentrations in duplicate of two products of bone metabolism – total deoxypyridinoline (tDPD) and osteocalcin (OC). tDPD is released into the bloodstream when bone is broken down and is used as a marker of bone resorption (hereafter bone resorption marker or bone resorptive activity). OC is released by the osteoblasts during bone deposition and is used as a marker of bone formation (here after bone deposition marker or bone deposition activity). Serum bone resorption and deposition markers were measured for each individual using commercially available ELISA kits (MicroVue Osteocalcin and MicroVue Total DPD, Quidel Corp., Santa Clara, CA) following manufacturer's instructions.

For statistical analyses, we classified sows based on parity (0-2 (n = 5), 3-4 (n = 5), or 5-6 (n = 5) previous reproductive events) and number of live offspring produced in the current litter (small 2-10 (n = 7), large 11-16 (n = 8). Sow age and parity were significantly correlated ($F_{1,61}$ = 266.26, R^2 = 0.816, $P < 0.0001$), therefore sow age was not included in the models.

We measured the effect of sow parity, live-born litter size, and live-born litter mass on serum concentrations of bone resorption and deposition markers over time, using a mixed model (PROC MIXED), with individual sow and sow mass at breeding as random effects. We selected an autoregressive (AR1) covariance structure based on AICC values and number of parameters included in the model. We ran separate models for gestation (prior to breeding to day of parturition) and lactation (day of parturition to weaning). We tested the effects of litter size and parity on total food consumed per individual during lactation in an ANOVA (PROC GLM). We tested relationships between the following: parity and litter size, litter size and mean individual offspring body mass, cumulative live-born offspring body mass (i.e. litter mass) at birth and parity or maternal body mass at birth, and parity and bone marker concentrations prior to breeding (day 1), using general linear models (PROC GLM). Analyses were conducted in SAS 9.1 (SAS Institute; Cary, NC USA).

Results

Live-born litter size ranged from 2 to 16 piglets. There was no relationship between litter size and mean maternal body mass at birth ($F_{1,13} = 0.04$, $P = 0.836$) or at weaning ($F_{1,13} < 0.001$, $P = 0.975$), nor was there a relationship between mean offspring mass at birth and mean offspring body mass at weaning ($F_{1,13} = 0.78$, $P = 0.394$). Neither parity nor litter size affected food intake during lactation ($F_{2,13} = 0.09$, $P = 0.915$; $F_{1,13} = 1.64$, $P = 0.236$, respectively), nor did these variables interact to affect food intake ($F_{2,13} = 1.02$, $P = 0.402$). There was a significant relationship between maternal mass prior to breeding and total live offspring mass at birth (Fig. 1) that was not affected by parity ($F_{2,13} = 0.26$, $P = 0.778$). There was no significant relationship between parity and total live offspring mass at birth ($F_{2,13} = 0.69$, $P = 0.530$).

There was a significant relationship between parity and rates of bone deposition ($F_{1,13}$ = 79.86, $P < 0.0001$); sows with the fewest prior reproductive events display higher concentrations of the bone deposition marker throughout gestation and lactation than those in the other parity classes (Fig. 2). No such relationship existed between parity and rates of bone resorption ($F_{1,13}$ = 0.94, $P = 0.369$).

Gestation

There was no significant relationship between day, litter size at birth, litter mass at birth, or parity and bone resorptive activity during gestation, nor were there any significant interactions between these variables at this time ($F \leq 4.58$, $P \geq 0.0726$ in all cases). Day of gestation significantly affected the concentration bone deposition markers ($F_{4,11}$= 4.44, $P = 0.0222$). Day of gestation also interacted with litter mass at birth ($F_{4,11}$ = 8.34, $P = 0.0024$), parity ($F_{7,11}$ = 7.35, $P = 0.0020$), and both litter mass at birth and parity ($F_{7,11}$= 7.96, $P = 0.0014$), to affect OC concentrations during gestation. Litter mass and the interaction between litter mass and parity also affected rates of bone deposition activity (litter mass, $F_{1,11}$ = 5.53, $P = 0.0383$)(litter mass * parity, $F_{1,11}$ = 6.98, $P = 0.0229$).

Lactation

During lactation, there was a significant effect of litter size, litter mass at birth and parity on bone resorptive activity (litter size: $F_{1,7.27}$ = 9.91, $P = 0.0154$; Fig. 3.; litter mass: $F_{1,7.39}$ = 10.27, $P = 0.0139$; parity: $F_{2,7.6}$ = 4.71, $P = 0.0467$; Fig. 2).There were also significant interactions between litter mass and litter size at birth ($F_{1,7.15}$ = 15.22, $P = 0.0056$), litter mass at birth and parity ($F_{2,\ 7.54}$ = 6.14, $P = 0.0262$) and between litter size at birth, litter mass at birth and

parity ($F_{1,7.92} = 10.12$, $P = 0.0131$). Day of lactation, parity and litter size at birth significantly interacted to affect bone deposition ($F_{1,8.02} = 6.44$, $P = 0.0347$).

Discussion

In swine, reproductive output, as represented by offspring body mass and litter size, affected bone metabolism, and maternal experience affected how mothers met the demand. Not surprisingly, bone metabolism changed throughout the course of gestation and lactation, reflecting changing mineral demands of developing young. During gestation, there was a positive relationship between litter mass and bone deposition activity while bone mobilization remained unchanged. This suggests that mothers are likely accumulating more mineral in their bones as a means of anticipating the elevated mineral demands of milk production associated with nursing large offspring, to support subsequent mineral demands of milk production. This has been observed in rats, in which mineral content of bone significantly increases during gestation and then decreases during lactation (Zeni et al., 1999).

Like most mammals, sows rapidly deposit calcium into their young during late gestation (Hansard et al. 1966). Calcium content is about 10.4 ± 1.3 g/offspring and 131 ± 12 g/litter at parturition, with about 50% of accumulation occurring during last 2 weeks of gestation (Mahan et al., 2009). This is similar for humans, where about 80% of the approximately 30 g of calcium deposited into human fetal skeleton is accreted during the last trimester. However, calcium transferred to young during lactation greatly exceeds gestation in most species. For example, the mean daily calcium deposition in the fetal skeletal averages 260 mg per day during the last trimester in humans, while up to 400 mg of calcium is lost daily during lactation (Kovacs 2005).

Litter size affected both bone deposition and bone mobilization during lactation, with generally greater concentrations of both bone deposition and bone resorptive markers being attributed to the production of more offspring (Fig. 2). Elevated bone metabolic activity with regards to both deposition and resorption indicates greater bone turnover in general. However, given the relationship between metabolic activity and number of offspring produced, it is likely that the processes of resorption and deposition remain uncoupled in order to allocate sufficient mineral to milk production, which increases with litter size in sows (Quesnel et al., 2007).

Elevated milk production would feasibly require greater maternal nutrient investment, however, litter size did not affect food intake in our study. Sows that produce large litters do not consume more feed during lactation than those producing smaller litters (Eissen et al., 2003; Eklou-Kalonji et al., 1999), but mobilization of maternal somatic stores of lipids and proteins does increases with litter size (Kim and Easter, 2001; Quesnel et al., 2007). This suggests not only that sows producing larger litters mobilize skeletal mineral to support elevated milk production, but that they rely more heavily on this mineral reserve than on dietary intake to support reproduction.

Maternal reproductive experience also affects bone metabolic activity during the reproductive bout. Fluctuations in bone weight and strength over the course of reproduction are greater in primiparous sows than 5^{th} parity sows (Giesemann et al., 1998), and, in our study, inexperienced females had higher concentrations of both bone deposition and bone resorptive markers compared to multiparous females. 5^{th} parity females also have larger, stronger and more mineralized bones than primiparous sows (Giesemann et al., 1998), and, in sheep and goats, bone loss during their second reproduction is less substantial that what is experienced during the first reproductive bout, even though milk production was greater (Liesegang et al., 2007). These

findings indicate that multiparous mammals are better equipped to meet with the mineral demands of offspring production, and that they may be more efficient at utilizing nutrients required during this time. Since there was no difference in offspring number or size relative to parity, it appears as if more experienced mothers continue to allocate sufficient resources to their young while minimizing potential costs to their own condition.

Limited resource availability can result in a reduction in litter size or offspring mass (eg. Geffen et al., 1996; Tannerfeldt and Angerbjorn, 1998), however, studies that manipulate dietary intake cannot address the effects of offspring production on maternal condition. When resources are abundant, such as in our study, we can begin to tease apart the influence that litter size and mass can have on maternal endogenous resource availability. Domestic sows have been artificially selected for production, and generally are slaughtered before they could incur costs associated with reproduction and old age; it follows that, in a sense, they have been selected to prioritize investment in offspring over self-maintenance. Indeed, the mass of individual offspring did not decline with increasing litter size, suggesting that mothers invested the same amount of resources into individual young regardless of the number produced. It is possible that developing offspring exert greater influence over the degree of mineral mobilized from the maternal skeleton relative to the mother, as indicated by the relationship between bone metabolic activity and reproductive output.

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Fig. 1. Relationship between sow body mass prior to breeding and the cumulative mass of the litter each produced at parturition ($R^2 = 0.475$, $F_{1,13} = 5.35$, $P = 0.0495$).

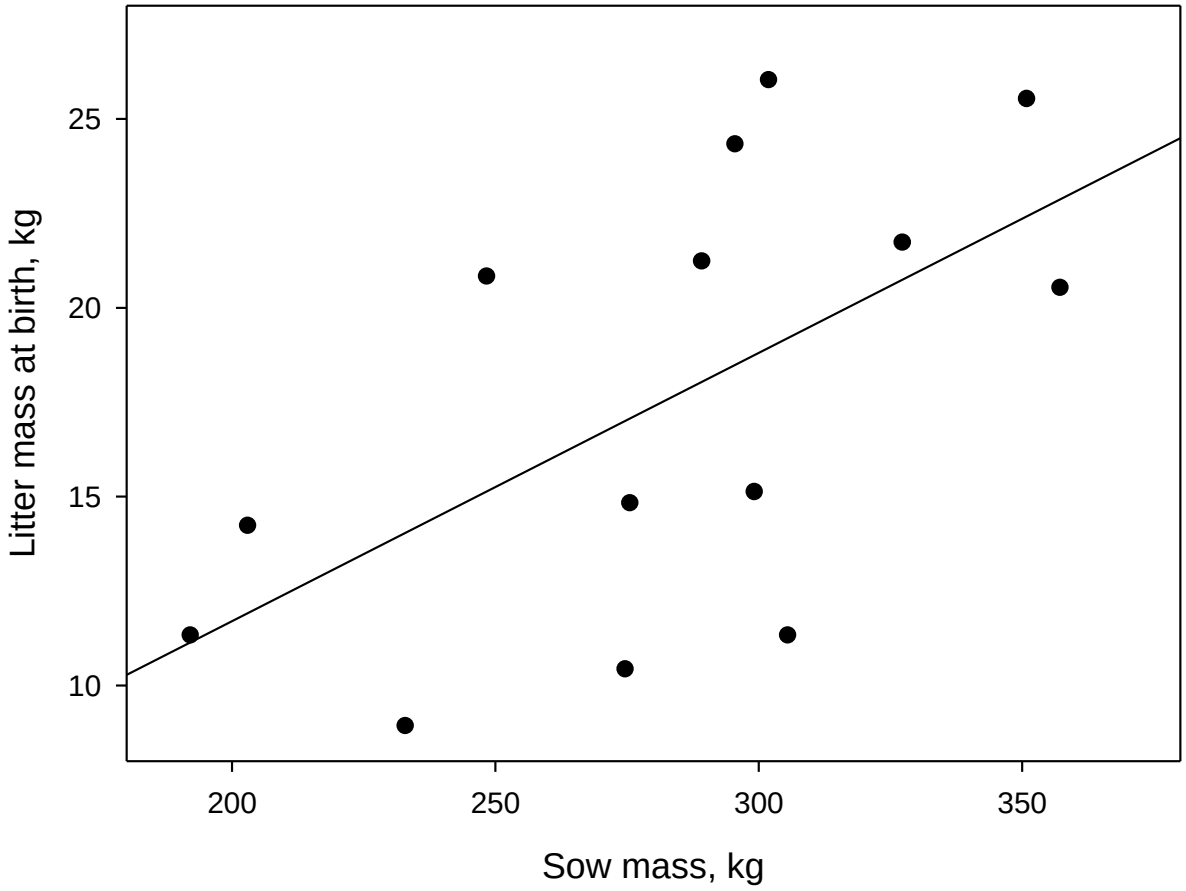


Fig. 2. Mean serum concentrations of tDPD (bone resorption) and OC (bone formation) during gestation and lactation for reproductively inexperienced (0 – 2 previous births), intermediate (3 – 4 previous births) and experienced (5 – 6 previous births) females.

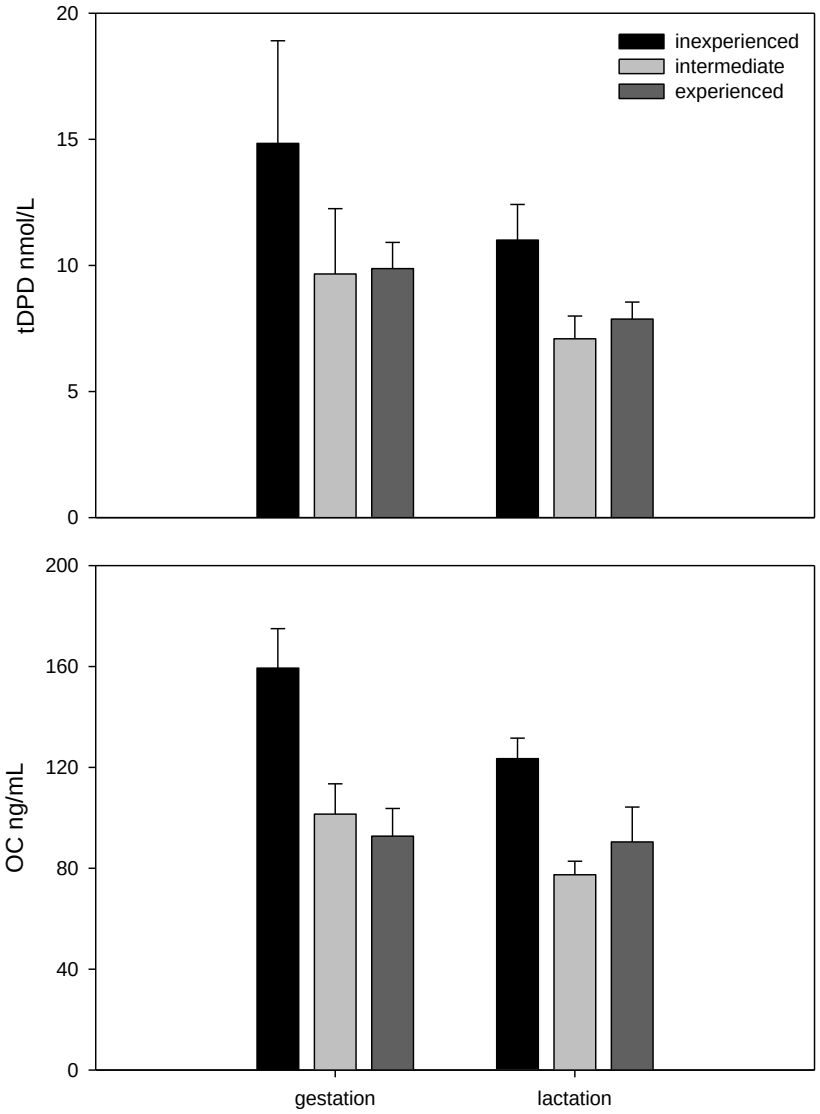
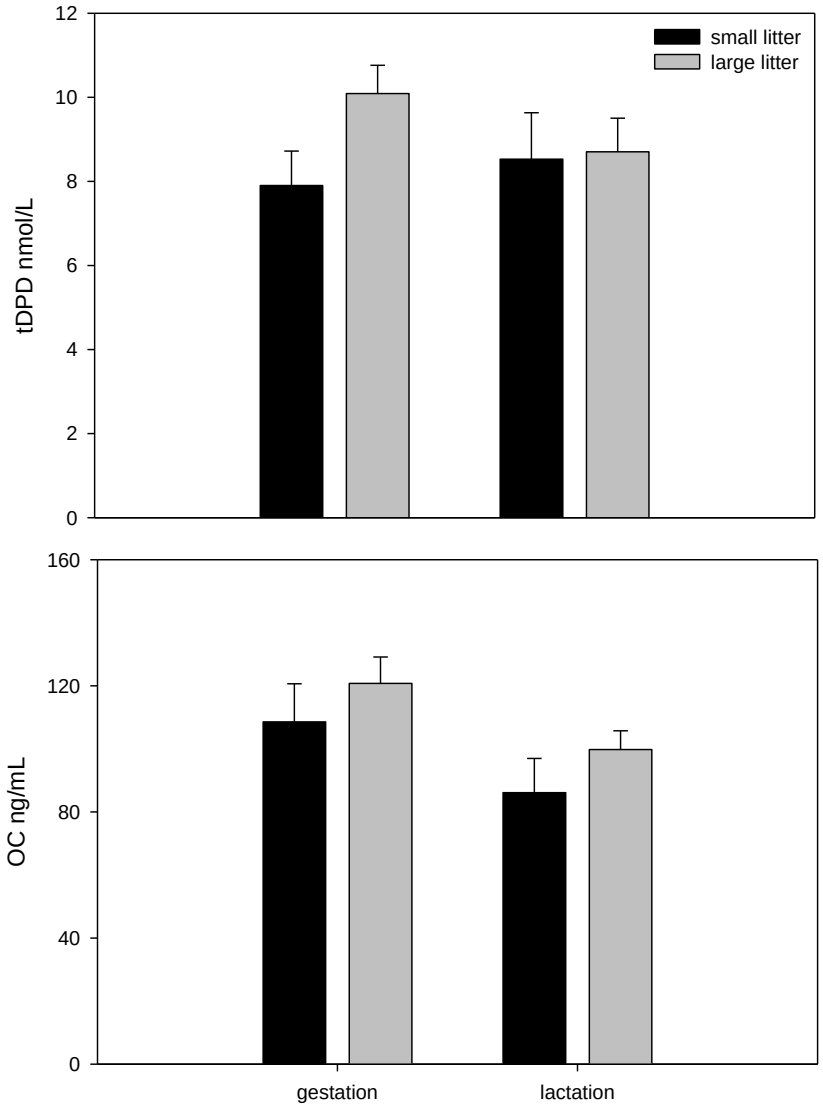


Fig. 3. Mean serum concentrations of tDPD (bone resorption) and OC (bone formation) during gestation and lactation for females that produced small litters (2 – 10 offspring) and large litters (11 – 16 offspring).



IV. Reproductive strategies to maximize lifetime reproductive success in response to calcium availability

Abstract

The production of offspring requires investment of resources derived from both the environment and maternal somatic reserves. As such, the availability these resources have the potential to limit the degree to which resources are allocated to reproduction. Theory and empirical studies have argued that mothers modify reproductive performance relative to exogenous resource availability and maternal condition, which is generally characterized by the amount of resources stored in somatic tissue, by adjusting size, number or sex of offspring produced. These relationships have classically been defined relative to availability of sources of energy; however, in vertebrates, calcium also plays a critical role in offspring production, as a considerable amount is required to support the development of an offspring's skeleton. We tested whether the availability of calcium influenced reproductive output by breeding female white-footed mice and providing them with a low-calcium or standard diet throughout their lives. We then compared maternal skeletal condition and reproductive output, characterized by offspring mass, offspring number and sex ratio of litters, between dietary treatments. Mothers on the low-calcium diet exhibited reduced skeletal condition at the end of their lives and produced smaller and strongly female-biased litters. We show that skeletal condition and calcium intake can influence sex ratio and reproductive output following general theoretical models of resource partitioning during reproduction.

Introduction

Life history theory predicts that mothers should optimize their reproductive success by partitioning resources in a manner that will afford them the greatest fitness; (Clutton-Brock, 1988; Clutton-Brock, 1991). To this end, maternal condition, maternal parity, and the interaction of these variables with the availability of nutritional resources in the animal's environment may impact the number and size of offspring produced. Under certain conditions, mothers may also adjust the primary sex ratio of their young.

Prior to and during reproduction, the interactions between somatic tissue storage by the mothers and the availability of nutrients in the animal's environment can be important determinants of the quantity of nutrients which can be devoted to production (but see Boutin, 1990). For example, poor body condition and food scarcity experienced by mothers during severe El Niño events is associated with reduced offspring body condition in California sea lions (Ono et al., 1987). Maternal body condition (residuals of body mass/skeletal size) is positively correlated with weaning mass under typically conditions in Columbian and Richardson's ground squirrels (Dobson and Michener, 1995; Skibiel et al., 2009). Greater maternal body fat stores and greater body fat utilization by mothers in polar bears and moose has also been correlated with greater weaning mass (Atkinson and Ramsay, 1995; Keech et al., 2000). And, the availability of food is positively correlated with litter size in Prairie voles and Columbian ground squirrels (Cole and Batzli, 1978; Dobson and Kjelgaard, 1985).

Trivers and Willard (Trivers and Willard, 1973) proposed that in polygynous species where reproductive success of males is more condition-dependent than that of females, mothers in good condition should produce more male progeny than mothers in poor condition. Yet, results of studies that address the relationship between maternal condition and primary sex ratio in

mammals are generally equivocal (Clutton-Brock, 1986; Rosenfeld and Roberts, 2004). Consistent support for the hypothesis that maternal condition can bias primary sex ratio in mammals can only be found when maternal condition is quantified around the time of conception (Cameron, 2004; Cameron and Linklater, 2007).

Most studies that examine the impact of maternal condition on reproductive output do so within an energetic context (see Boutin, 1990; Clutton-Brock, 1986; Rosenfeld and Roberts, 2004; Speakman, 2008; for reviews). Following this trend, individual differences in maternal condition are frequently quantified based on direct or indirect estimates of endogenous stores of fat, representing the body's primary energy reserve used to support cellular metabolism. However, condition is best described as a measure of the functionality of cellular processes in the body that encompass a broad array of genetic and phenotypic components (Hill, 2011), and these processes are not necessary associated with energy utilization. In addition to energy, a substantial amount of calcium must also be partitioned to mammalian offspring to support skeletal development and mineralization. Available calcium is traded-off between maternal skeletal stores and developing bone in their young. Thus, processes supporting maternal and offspring skeletal growth and integrity must also be considered to be vital components of condition (Kovacs, 2005; Kovacs and Kronenberg, 1997).

Maternal bone loss during gestation and lactation has been observed in several species of mammals (Liesegang et al., 2007; Ott et al., 1999; Wysolmerski). Although bone loss increases the risk of sustaining a fracture (Currey, 1969, 1984) and thus can be assumed to decrease a mother's probability of survival, mothers commonly maintain mineral allocation to their young by supplementing ingested calcium with calcium from their own skeleton (Boelter and Greenberg, 1943; Bowman and Miller, 2001; Gruber and Stover, 1994; Kalkwarf and Specker,

2002; Kovacs, 2005; Kovacs and Kronenberg, 1997; Prentice, 2000; Zeni et al., 2003). Thus, a trade-off likely exists between maternal skeletal condition and offspring production in mammals. Small mammals may be more susceptible to this interaction for three reasons. First, food, and thus nutrient intake, in small species may be limited by gastrointestinal capacity to absorb nutrients and the body's ability to partition available nutrients to offspring (Speakman, 2008). Second, small mammals possess proportionately smaller skeletons, and thus smaller calcium reserves, than larger mammals (Hood et al., 2006; Prange et al., 1979). Finally, small mammals transfer relatively large amounts of calcium to their offspring, based on high milk outputs (Oftedal, 1984) and milk calcium concentrations that are comparable to other mammals (Studier and Kunz, 1995).

Few studies have addressed how calcium intake affects reproductive output in mammals outside of addressing offspring skeletal characteristics. It has been shown that big brown bats (*Eptesicus fuscus*) produce larger offspring relative to maternal mass when fed a calcium supplement (Booher, 2008), and hypocalcemia has been associated with diminished litter size and female fertility in rats (Johnson and DeLuca, 2002; Rosenfeld and Roberts, 2004). With regard to sex ratio, female rodents tend to produce less males when stressed by nutritional deficiencies (Rosenfeld and Roberts, 2004), however the potential impact of calcium deficiency on sex ratio has not been previously investigated.

Given the cost incurred by the maternal skeleton associated with offspring production and that skeletal condition reflects both exogenous and endogenous calcium availability, we hypothesized that mammals adjust reproductive output relative to exogenous calcium availability and endogenous calcium stores in the skeleton. Specifically, we predicted that females with low calcium intake and lower bone mineral content, as indicated by architectural characteristics of

bone at key sites of mobilization, would give birth to fewer and/or smaller young and the primary sex ratio of the young would be skewed toward females. To test this, we manipulated calcium intake of captive white-footed mice (*Peromyscus leucopus*) to assess the relationship between dietary calcium, maternal skeletal condition, and parameters of reproductive performance over an individual's lifetime. White-footed mice lose bone over the course of gestation and lactation, and bone loss is intensified when mothers are consuming a low-calcium diet (Chapter I). White-footed mice also allocate less calcium to individual offspring when dietary intake is reduced (Chapter II), thus making them a good potential model for testing the Trivers-Willard hypothesis within the context of calcium utilization. We also took into account other factors that may influence reproductive output, namely maternal age, parity and mass, to determine their impact as well as whether calcium intake interacted with these factors to affect reproductive performance.

Materials and Methods

We obtained 15 nulliparous female and 8 male white-footed mice from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC USA) and maintained them at an animal facility at Auburn University (Auburn, AL USA) for the duration of the study. Females were housed in pairs in 29 x 19 x 12.5 cm polypropylene rodent enclosures (Lab Products, Inc., Seaford, DE USA) at 25^{o}C on a 16:8 h light cycle. We provided females with *ad-lib* water and access to one of two custom manufactured diets that differed only in calcium content (see Chapter II, Table 1; Harlan Teklad, Madison, WI USA). Seven females received a low-calcium diet (0.10% calcium) and 8 females received a standard diet (0.85% calcium), which provided recommended calcium intake for reproductive mice (Harlan Teklad, pers.

comm.) We provided females with 4-5 breeding opportunities over a period of 75 weeks by placing a randomly selected male in their enclosure for 14 days, ensuring that females were never introduced to the same male more than once. Pups were counted, sexed, and weighed 7 days post-parturition, and were weighed again every 7 days after that until fully weaned (day 28). We weighed mothers every 7 days throughout the study, with the exception of the week following parturition.

Following Charnov et al. (2007), we calculated lifetime reproductive effort (LRE) of each female as the product of total number of offspring produced and mass of offspring at independence relative to mother's mass at first reproduction:

LRE = total offspring produced x (offspring mass at independence / adult mass at first reproduction)

where offspring mass at independence was considered to be mass at day 21, and adult mass at first reproduction as the last recorded mass (within 14 days) prior to first successful fertilization. Although not completely weaned at this time, we observed pups between the ages of 21-28 days consuming solid foods, thus, we used the mass of 21 day old pups in order to more accurately represent maternal investment.

We euthanized mothers at either 85 or 100 weeks of age to simulate average lifespan of two years, and excised the left femur, left humerus and a section of lumbar vertebrae to quantify skeletal condition. We measured relative bone volume, trabecular number, trabecular thickness and trabecular separation of femurs and relative bone volume of lumbar vertebrae using micro-computed tomography (MicroCT 40, Scanco Medical, Bassersdorf, Switzerland). We calculated bone flexural strength of femurs and humeri on a custom made 3-point bending fixture using

monotonic axial displacement, using an 858 MiniBionix Material Testing System with a 100 N load cell (MTS Systems Corp., Minneapolis, MN USA). The contacts of the 3-point apparatus were set at a span of 8 mm for humeri and 10 mm for femurs, and a cross head speed of 0.05 mm/sec was used to break the bone mid-shaft.

We compared maternal bone characteristics between dietary treatments using student's t-tests. For femur characteristics, we corrected the α-value for multiple comparisons using a sequential Bonferroni for correlated variables. These values were calculated using the Qualitative Skills webpage (http://www.quantitativeskills.com). We used a general linear model (PROC GLM) to determine if maternal mass prior to fertilization affected litter size, mean pup mass at day 7 or mean pup mass at day 21. We used a two level nested ANOVA, with pup nested within mother, to determine if pup mass at day 7 and day 21 varied between sex, and if maternal diet interacted with sex to affect mass (PROC GLM). We used mixed models (PROC MIXED), with mother specified as a random effect and parity as a repeated variable, to test if calcium content of maternal diet, age of mother at parturition, body mass of mother prior to fertilization or parity affected the proportion of males produced per litter, litter size, or mean offspring mass at day 7 and day 21. We selected an autoregressive covariance matrix (AR1) for these analyses based on AICC values. We used a student's t-test to compare calculated LRE and total offspring mass produced for each mother between diets. One of the females assigned to the low-calcium diet died during the study and therefore is not included in analyses. Unless otherwise specified, statistical analyses were performed in SAS v9.2 (SAS Institute Inc., Cary, NC, USA). All means are given ± standard error.

Results

Maternal bone characteristics

Vertebral cortical bone volume was significantly lower for females that had consumed the low-calcium diet (t_{13} = 2.78; Table 1) and there was a trend suggesting that femoral cortical bone volume could also be lower in females that consumed the low-calcium diet (t_{12} = 2.11; Table 1) but this was not significant with Bonferroni correction. There was no significant difference between diets for any of the trabecular bone measurements of the femur (Table 1).There was no difference between diets in force required to break mothers' femur or humerus (Table 1).

Reproductive output

Mothers on the low-calcium diet produced a significantly smaller proportion of males per litter on average (0.194 ± 0.104) than females on the standard diet (0.506 ± 0.091; $F_{1,24}$ = 5.47, P = 0.036; Fig. 1). There was no difference in body mass of male and female pups of mothers consuming the standard or low calcium diet at day 7 or 21 or pups ($F \leq 1.60$, $P \geq 0.21$ for all models).

Maternal mass prior to fertilization had no effect on litter size or mean pup mass at day 7 or day 21 ($F_{1,9}$ = 0.84, P = 0.39; $F_{1,8}$ < 0.001, P = 0.99; $F_{1,8}$ = 0.01, P = 0.91; respectively), nor did diet interact with maternal mass to affect these variables ($F_{1,9}$ = 3.74, P = 0.10; $F_{1,8}$ = 0.04, P = 0.84; $F_{1,8}$ = 0.21, P = 0.67; respectively).

Age of mother at parturition and calcium content of maternal diet each had a significant effect on litter size (Age: $F_{1,24}$ = 5.72, P = 0.033; Diet: $F_{1,24}$ = 5.38, P = 0.037), but did not significantly interact to affect litter size ($F_{1,24}$ = 4.24, P = 0.060). Females on the low-calcium diet produced, on average, 2.22 ± 0.36 pups per litter, whereas females consuming the standard

diet produced an average of 2.69 ± 0.28 pups per litter. Litter size decreased with age. Calcium content of diet, maternal age at parturition and parity had no significant effect on mean pup mass at day 7 or day 21 ($F \leq 2.04$, $P \geq 0.17$ for all models, Table 1).There was no significant difference between diets for calculated LRE ($t_{13} = 0.99$, $P = 0.34$; Table 1).

Discussion

Given that calcium is vital for offspring skeletal development in mammals, it should follow that the availability of this resource influences reproductive performance. An individual's fitness is determined by its own survivorship and fecundity, as well as the survivorship and fecundity of its offspring. Thus to optimize fitness, mothers must adjust their nutritive effort based on both their endogenous condition and the availability of exogenous resources. Here we have shown that nutritive allocation decisions are not only made base on the availability of energy but also with regard to available calcium.

Females subjected to a low-calcium diet throughout their lives displayed a long term reduction in bone volume. Diminished bone volume can be interpreted as a reduction in skeletal condition, as well as a reduction in calcium reserves available for allocation to developing offspring. These females produced smaller litter sizes and bore relatively fewer male pups that female consuming a diet believed to be nutritionally complete; however pup mass at birth and weaning and lifetime reproductive effort were not affected by maternal calcium intake.

Thus, if maternal skeletal condition is used as a proxy for maternal condition, our results support the Trivers-Willard hypothesis (Trivers and Willard, 1973). Maternal skeletal condition at the end of the mother's lifetime had significantly deteriorated for mothers consuming a calcium-deficient diet relative to those consuming the standard diet. Although mammals are

generally capable of recouping lost bone after a reproductive event (eg. Bowman and Miller, 1999; Miller et al., 2005), the difference in skeletal condition that we observed suggests that bone loss experienced over the course of reproduction can have a sustained, long-term effect on the mother's skeleton.

A main assumption of the Trivers-Willard hypothesis is that mothers in better condition are able to, and do, allocate more resources to their offspring. In human studies, there is some evidence that maternal calcium intake is positively related with offspring bone mineralization (Koo et al., 1999; Raman et al., 1978). In a concurrent study on white-footed mice, we found that mothers did indeed allocate less calcium to individual offspring when their dietary calcium intake was reduced (Chapter II). Additionally, the mid-shaft width of femurs of pups from mothers on the standard diet was greater than that of pups produced by mothers on the low-calcium diet (Chapter II), which could affect offspring bone strength/resistance to fracture into adulthood. Thus, pups receiving more calcium during development may experience long term advantages in both survival and reproduction relative to those that are calcium limited.

Mothers that consumed the low-calcium diet produced female-biased litters, whereas the primary sex ratio of mothers that consumed the standard diet was approximately 1:1. In this case, maternal skeletal condition represents endogenous calcium reserves that are presumably available for offspring investment, much like typical indices of condition represent energetic reserves. In mammals, the effect of maternal condition on primary sex ratio has only been consistently demonstrated when quantifying condition at conception (Cameron, 2004; Cameron and Linklater, 2007). Although not directly tested, since calcium intake has both long- and short-term effects on the bone characteristics of reproductive white-footed mice, it is feasible that skeletal condition at conception also differed between diets.

Interestingly, a similar reduction in litter size and number of males produced was found when laboratory mice were fed a low-fat diet, which was independent of maternal mass (Dama et al., 2011; Rosenfeld et al., 2003). Fat and bone are connected mechanistically through a variety of pathways (reviewed in Reid, 2010). As such, this similar effect may be indicative of a potential target for studies on the mechanisms underlying sex ratio and offspring production in mammals. The production of female-biased litters may also be a response to nutritional stress in general, as a decrease in number of males produced by rats was observed as maternal dietary salt intake increased (Bird and Contreras, 1986).

Rosenfeld and Roberts (2004) discussed several proposed mechanisms that link maternal diet and nutritional condition to skewed sex ratios in mammals. Briefly, the sperm of one sex may be differentially affected by the reproductive tract milieu in such a way as to influence motility or fertilization ability, or reproductive tract conditions may differentially affect the embryonic development of one sex over the other. Among its many functions, calcium plays a significant role in affecting sperm motility, oocyte activation, fertilization, sex differentiation of germ cells, embryo activation, and differentiation of embryonic stem cells in mammals (Cuthbertson et al., 1981; Ducibella et al., 2002; Hanover et al., 2009; Heytens et al., 2008; Miyazaki et al., 1993; Ramalho-Santos et al., 2009; Spiller et al., 2009). Therefore it is possible that any of the above mentioned functions could be mediated by maternal calcium availability.

Considerable variation in sex ratio exists within the *Peromyscus* genera, however, most species produce male biased litters (reviewed in Terman and Sassaman, 1967)). Wild trapped white-footed mice exhibited an approximately 1:1 ratio (Kaufman and Kaufman, 1982). However, season and maternal mass have been associated with skewed sex ratios, with male biased litters produced in spring, when females are heavier, and female biased litters produced in

fall, when mothers have less mass (Goundie and Vessey, 1986) which likely reflects seasonal fluctuations in food abundance or a depletion of somatic resources over the reproductive season. In our study, females on the low-calcium diet were heavier than those on the standard diet. This may be due to females attempting to compensate for low calcium content of their food by consuming more food overall; however, given the disparate calcium content between the two diets, it was unlikely that females on the low-calcium diet approached calcium intake of females on the standard diet.

For rodents and other vertebrates, variation of calcium availability can occur both geographically and temporally; soil may be acidified, resulting in low calcium content of invertebrate prey items (eg. Graveland and Wal, 1996), and shed antlers, which may serve as a calcium supplement for gnawing rodents, may not be available year round. We can conclude that in white-footed mice sources of variation in dietary calcium availability during reproduction, such as these, can influence primary sex ratio and litter size, and that a mother's skeletal condition should be viewed as a key indicator of maternal condition that can influence reproductive strategies. Females that experienced calcium deficiency over the course of their life produced smaller, female-biased litters, which would theoretically limit their contribution to the overall population relative to females that were consuming sufficient calcium.

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Table 1. Maternal bone characteristics for females consuming a low-calcium or standard diet.

		Low-Ca	**Standard**	
	Structure	$\bar{X}$ **± SE**	$\bar{X}$ **± SE**	***P***
Cortical bone volume (BV/TV%)	Femur	0.482 ± 0.109	0.534 ± 0.093	0.028‡
	Vertebrae	0.155 ± 0.008	0.182 ± 0.005	0.019
Trabecular bone volume (BV/TV%)	Femur	0.0123 ± 0.0095	0.0177 ± 0.0086	0.68
Trabecular number	Femur	1.41 ± 0.121	1.37 ± 0.118	0.86
Trabecular thickness (μm)	Femur	0.0350 ± 0.0068	0.0447 ± 0.0049	0.27
Trabecular separation (μm)	Femur	0.775 ± 0.0626	0.789 ± 0.0681	0.88
Breaking force (N)	Femur	14.2 ± 0.78	15.9 ± 0.65	0.13
	Humerus	14.3 ± 0.48	14.1 ± 0.84	0.86

‡ Bonferroni corrected $\alpha = 0.020$

Table 2. Lifetime reproductive output of white-footed mice consuming a low-calcium or standard diet.

	Low-Ca $\bar{X}$ **± SE**	**Standard** $\bar{X}$ **± SE**	***P***
Mean litter size	2.22 ± 0.36	2.69 ± 0.28	0.037*
Total offspring produced	3.88 ± 0.93	5.71 ± 1.22	0.248
Mean pup mass (g), day 7	4.02 ± 0.29	4.30 ± 0.30	0.388
Mean pup mass (g), day 21	8.78 ± 0.4	10.0 ± 0. 6	0.220
Males per litter	0.194 ± 0.104	0.506 ± 0.091	0.036*
Lifetime reproductive effort	1.88± 0.548	2.55 ± 0.38	0.330
Cumulative offspring mass (g), day 21	30.4 ± 7.9	55.3 ± 9.9	0.075

Lifetime reproductive effort (LRE) based on Charnov et al. (2007).

Fig. 1. Proportion of males in litters produced by females consuming either a low-calcium or standard diet. The dashed line represents a 1:1 sex ratio (i.e. 50% males).