The evolution of milk composition and lactation strategy of the Columbian ground squirrel, *Urocitellus columbianus*

by

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Abstract

The evolution of parental care improved the efficiency of the reproductive process, allowing for the production of fewer offspring with a higher probability of survival. This adaptation is exemplified in the taxon named for their unique and efficient mechanism of nourishing young following parturition. Lactation and milk production is a key feature of the mammalian reproductive strategy contributing to maternal and offspring fitness. Because the quantity and quality of care provided is a balance between maternal cost and benefit, identifying factors contributing to inter and intra-specific variation in lactation effort as well as the costs and benefits involved can provide keen insight into the evolution of mammalian life history strategies. Herein, I determined factors influential in the evolution of milk composition and examine the causes, benefits and costs associated with lactation effort in the Columbian ground squirrel, *Urocitellus columbianus*.

First, I examined factors contributing to the evolution of the diverse nutritional composition of milks observed across mammals using a phylogenetic comparative approach. The biome inhabited, length of the lactation period, and maternal diet were correlated with milk composition. Second, I described the composition of milk produced by Columbian ground squirrels. The concentration of most milk constituents changed over the course of lactation with most exhibiting a distinct peak around 19 days postpartum. Columbian ground squirrel milk was relatively low in lipid concentration but high in protein and calcium concentration and the proportion of energy from protein. Third, I assessed relationships between maternal characteristics and milk composition and impacts on offspring. Variation among females in milk fat, sugar, and protein concentration and energy density was not associated with differences among females in size or condition. Females giving birth around the median parturition date produced milk that was higher in fat concentration and energy than females giving birth at other times. Milk fat, energy, and sugar concentration had a positive effect on offspring survival over-winter. Finally, I examined the proximate and ultimate costs of lactation. Females raising augmented litters had greater rates of energy expenditure, indicating an energetic cost to lactation. Energy expenditure was not related to female survival over-winter or her fecundity the next breeding season, although pups of augmented litters were lighter at weaning, had slower growth rates, and a lower probability of survival over-winter. These results do not support the predictions associated with the cost of reproduction tradeoff.

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INTRODUCTION

Parental care is a fascinating and critical component of life history strategies that evolved as an adaptation to maximize propagation of parental genes across subsequent generations (Gross 2005). Patterns of parental care vary widely across the animal kingdom where differences among species can be found in nest or oviposition site selection and preparation, incubation, gestation, and lactation behaviors, territory defense, protection of progeny from predators and conspecifics, among others (Clutton-Brock 1991, Rosenblatt and Snowdon 1996). Parental care benefits offspring in a multitude of ways including promoting growth, development, learning, social relationships, and ultimately survival and future reproductive success (Clutton-Brock 1991). Hence, parental care can be pivotal for parental and offspring fitness. Although beneficial to offspring, parental care often entails a cost to the provider. Factors that have led to the evolution of parental care include, the ratio of offspring benefits to parental costs of providing the care, other aspects of the species life history, offspring behaviors that influence parental care decisions, environmental conditions, and resource availability and predictability, all of which are likely complexly interconnected (Stearns 1976, Maynard Smith 1977, Winkler 1987, Clutton-Brock 1991, Rosenblatt and Snowdon 1996).

In mammals, perhaps one of the most prominent parental care behaviors is milk production and the allocation of vital nutrients and energy to developing young. In mammals, nutritional provisioning occurs at two critical life history stages, gestation and lactation. During gestation nutrients are transferred to the developing embryo through an allantoic or vitelline placenta for initial tissue and organ growth (Griffiths 1978, Gilbert 2003) whereas during lactation nutrients critical for continued tissue, structural and organ growth and development are transferred to young through maternally synthesized milk (Jenness 1985). Lactation is particularly interesting for many reasons. First, the synthesis of milk within the mammary gland is a unique characteristic of the class Mammalia (Gregory 1910). Second, milk is produced within the maternal body from the building blocks of macronutrients that can be acquired and stored prior to their use, allowing neonatal nutrient intake to be buffered from fluctuations in resource availability (Pond 1977, 1984, Oftedal 2011). Third, lactation is the most energetically expensive phase of mammalian reproduction, due in large part to the high metabolic demands of milk synthesis (Gittleman and Thompson 1988). Finally, nutrient transfer during lactation not only has immediate effects on offspring physiology but can permanently alter offspring phenotype (Lucas 1991, Palou and Picó 2009, Hinde and Capitanio 2010).

Our understanding of the mammary gland, the biochemical properties of milk, and the biosynthesis of milk has grown tremendously in the past 4 to 5 decades, largely attributable to the animal science industry's efforts to maximize milk quality and quantity and the biomedical field. Although this has led to the formulation of numerous methods for determining milk composition and advanced our understanding of lactation in general, lacking is an exploration of lactation biology from an evolutionary perspective. Thus, there is much to be gained from empirical studies on lactation both within and among species that will contribute to our understanding of the evolution of life history strategies in mammals.

The first step towards conceptualizing lactation biology within an evolutionary framework is determining the causes of variation among species in the diversity of milks observed. The nutritive composition of milk varies drastically among species and the factors contributing to this variation have yet to be elucidated. For example, black rhinoceros, *Diceros bicornis*, milk consists of 0.2% fat whereas hooded seal, *Cystophora cristata*, milk is 61% fat (Oftedal 1984, Oftedal and Iverson 1995). Milk sugar content ranges from trace concentrations (less than 0.05%) in some species of phocid seals to 14% in the long-nosed potoroo, *Potorous tridactylus* (Oftedal 1984, Oftedal and Iverson 1995). Hypotheses considering differences in ecologies and life histories among species have been advanced to explain why certain mammalian groups are more similar in milk composition than others but rigorous testing of these hypotheses in a phylogenetically informed comparative analysis is lacking. Without considering shared evolutionary history it is impossible to know with any confidence whether similarities in the nutritive composition of milk are due to shared life history strategies or simply evolutionary relatedness (Blackburn 1993).

The second step towards conceptualizing lactation biology within an evolutionary framework is understanding the causes and consequences of intra-specific variation in lactation, and costs associated with milk production within species. Traditionally, milk composition was thought to be relatively invariant among individuals within a species while milk yield was thought to change according to reproductive demand, changes in resource availability, etc (Bohstedt 1972, Sampson and Jansen 1984). In this manner, the relative proportions of nutrients transferred to the young remain stable while the total amount of nutrients delivered can vary. A growing body of evidence, however, is challenging this assertion, demonstrating that differences among females in key life history characteristics, such as litter size, condition and timing of reproduction can have vital impacts on milk composition with implications for maternal and offspring fitness. For example, in the common marmoset, *Macaca mulatta*, heavier females with larger body fat stores produce milk richer in fat content, resulting in faster infant growth rates (Tardif et al. 2001). In Iberian red deer, *Cervus elaphus hispanicus*, fat and energy content of milk is associated with timing of reproduction with an effect on offspring growth rates (Gomez et al. 2002). Although these studies demonstrate relationships between maternal and offspring phenotype due to milk composition, few take it one step further to determine if these relationships actually result in fitness differences among females.

As with any other parental care behavior, nutritional provisioning of young not only benefits the young in terms of growth, development, and survival, but can also entail a cost to the provider (Trivers 1972). Resources invested in producing milk during lactation, for example, can decrease the availability of resources that can be allocated to other vital processes such as maintaining immune function, skeletal integrity, body mass, etc. This is the basic premise of the cost of reproduction, a life history tradeoff that states that current reproductive effort may come at a cost to maternal survival or future fecundity (Williams 1966a, Williams 1966b, Bell 1980). More recently, researchers are attempting to address underlying physiological costs, such as energetic costs, that might underpin traditional life history tradeoffs (Speakman 2008). Although energy intake and expenditure are often associated with varying reproductive demand (Randolph et al.

1977, Glazier 1985, Hammond and Diamond 1992, Humphries and Boutin 2000, Johnson et al. 2001), it is unclear if this underlies an ultimate cost to reproduction.

My hope through these studies is to provide a framework for contextualizing lactation from an evolutionary perspective and to provide further insight into the evolution of a key component of a mammal's life history strategy, the nutritional provisioning of young through lactation. In the studies herein I first test hypotheses regarding the evolution of the variation in milk composition observed across mammals. Second, I address the causes and consequences of intra-specific variation in Columbian ground squirrel milk and the energetic and ecological costs associated with lactation. Columbian ground squirrels are relatively small-bodied and long-lived rodents with an interesting reproductive ecology and life history. Columbian ground squirrels inhabit alpine and subalpine meadows in the northwestern United States and southwestern Canada (Elliott and Flinders 1991). Average longevity of female Columbian ground squirrels is 5.5 years (Neuhaus et al. 2004). Females produce one litter per year and have an average litter size of 3 pups (Murie et al. 1980). This species hibernates obligatorily 8-9 months of the year leaving an active season of only 3-4 months in which to breed (Murie and Harris 1982). Columbian ground squirrels fast over-winter and thus rely primarily on somatic fat stores for energy during hibernation (Murie and Boag 1984, Young 1990, Dobson 1992). Lactation lasts approximately 27 days, during which time the only source of nutrition for pups is through the mother's milk (Murie, 1992). Unlike many other hibernators, the young are weaned just a few weeks before hibernation commences (Dobson and Murie 1987, Dobson et al. 1992). Furthermore, maternal investment during lactation is exceedingly important to offspring growth and to the over-winter survival of juveniles (Skibiel et al. 2009). Given the length of investment in lactation and the short amount of time post-weaning to gain fat mass, females are physiologically tested every year and constraints of hibernation on lactation effort are expected.

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CHAPTER ONE

THE EVOLUTION OF THE NUTRIENT COMPOSITION OF MAMMALIAN MILKS

Abstract. Parental care constitutes behaviors that are beneficial to the offspring and varies widely among taxonomic groups. In mammals, nutrient allocation during lactation is a critical component of parental care as milk intake promotes juvenile tissue and organ growth, development, and survival, and hence maternal and offspring fitness. The specific composition of milk varies widely across mammalian species and is thought to have arisen via selection pressures associated with developmental stage at birth, habitat, and diet, among others. These hypotheses have been put forth based on observations or cross-species comparisons that do not take into consideration past evolutionary history. We conducted the largest comparative analysis of milk composition to date accounting for phylogenetic relationships among species in order to understand which factors were most influential in the evolution of the diverse composition of milks produced by extant mammals. In this study, strong phylogenetic signal (the tendency for related species to resemble each other) was apparent for all milk constituents analyzed (fat, protein, dry matter, and gross energy). After controlling for phylogeny, we found that diet, the length of the lactation period, and whether a species is terrestrial or aquatic explained the greatest amount of variation in milk composition. Our results indicate not only the importance of accounting for phylogeny in comparative analyses but suggest that production of milk with a specific nutritional profile evolved largely as a function of the

combined effects of maternal nutrient intake, the length of time neonates require maternal provisioning, and habitat occupation.

INTRODUCTION

Few behaviors are as complex and widely varying in their patterns among species than parental care, an important adaptation that has allowed species to thrive in diverse habitats and under variable environmental conditions by promoting the propagation of genes into future generations (Rosenblatt and Snowdon 1996). Species vary in many components of parental care including which parent gives the care, the quantity of time, energy, and resources invested, the type of parental care given, and the length of parental care (Clutton-Brock 1991). In mammals, one the of most well-studied parental care behaviors is maternal nutritive investment in young, which is essential for offspring growth and survival, yet relatively little is known about the evolution of this important aspect of parental care. Nutritive investment in offspring occurs both in utero and during lactation in mammals and supports development and functionality of organ systems, structural growth, and neurological development. Because the proportion of nutrients that are provisioned during lactation is typically far greater than in utero (Gittleman and Thompson 1988), nutritional provisioning during lactation is easier to quantify than nutrient transfer in utero, and nutritional provisioning during lactation varies widely among species, understanding the evolution of lactation and the nutritional composition of milk in particular is likely to give us valuable insight into factors contributing to the evolution of offspring provisioning.

The specific types and concentrations of nutrients found in milk vary both within and among species. Within species, milk fat concentration appears to be the component that varies most among females and milk composition is associated with maternal characteristics such as body condition or size (Nommsen et al. 1991, Doreau et al. 1992, Georges et al. 2001, Tardif et al. 2001, Hinde 2007) and parity (Nommsen et al. 1991, Dewey 1997), timing of reproduction (Landete-Castillejos et al. 2000), offspring sex (Hinde 2009), litter size (König et al. 1988, Fiorotto et al. 1991, Rogowitz and McClure 1995, Rogowitz 1998, Tardif et al. 2001), and stage of lactation (Oftedal 1984, Oftedal and Iverson 1995). Across species, substantial variation exists in the proximate constituents of milk (*i.e.* protein, fat, sugars) and milk energy density. For example, fat varies from 0.2% in the black rhinoceros, *Diceros bicornis*, to 60% in some species of Phocid seals (Oftedal and Iverson 1995), milk sugars are virtually non-existent in some pinnipeds and greater than 11% in some Diprotodont marsupials (Oftedal 2000), and protein varies from just slightly >1% in some primate species to almost 16% in the eastern cottontail, *Sylvilagus floridanus* (Oftedal and Iverson 1995).

The adaptive significance of this inter-specific variation is unclear (Oftedal et al. 1993), but the specific composition of milk produced by a species has been postulated to have been selected for on the basis of the immunological, thermoregulatory, osmoregulatory, or nutritive needs of the young (Payne and Wheeler 1968, Oftedal and Jenness 1988, Peddemors et al. 1989, Blackburn 1993, Kunz et al. 1995, Oftedal and Iverson 1995, Tilden and Oftedal 1997). As such, variation among species in factors such as life history, diet, and habitat are hypothesized to be associated with inter-specific differences in lactation strategies (Oftedal 1984, Oftedal and Iverson 1995, Kunz and Hood 2000, Hinde and Milligan 2011). Unfortunately, our understanding of the role of these factors in the evolution of the diverse composition of milks observed across mammals is currently limited.

Comparative studies of milk composition have been largely qualitative, with species compared based on similar characteristics, or quantitative but neglecting to incorporate species relatedness in statistical analyses. These approaches are problematic because related species tend to resemble each other and thus confound interpretation of potential ecological, morphological, behavioral, and/or physiological explanations for inter-specific variation (Blackburn 1993). Furthermore, not accounting for evolutionary history violates the assumption that data are independent, which artificially inflates the degrees of freedom and increases type I error rates (Felsenstein 1985, Garland and Adolph 1994, Garland et al. 2005). Although more recent inter-specific comparisons of milk composition have accounted for phylogeny, these studies have been based on restricted phylogenetic groups, such as within the order Primates (Hinde and Milligan 2011), within pinnipeds (Schulz and Bowen 2005), or within eutherian mammals (Langer 2008). Furthermore, some of these comparative studies (Langer 2008, Hinde and Milligan 2011) include milk data that were collected at different times within the lactation period. Because temporal variation in milk composition exists, comparing milks of different species taken from different time periods confounds inter-specific variation with intra-specific temporal variation (Oftedal 1984, Oftedal and Iverson 1995). Here we use a phylogenetic approach to test hypotheses proposed to explain differences among mammals (including eutherian, prototherian, and metatherian groups) in milk composition at mid lactation.

Hypotheses and predictions

We consider several hypotheses previously advanced to explain the evolution of milks across mammals. In addition, we tested predictions modified from existing hypotheses based on our own ideas.

Body size. Relationships between milk composition and body size are equivocal in the literature. After accounting for phylogeny, Langer (2008) found no relationship between dry matter content of milk and body mass among 62 species of eutherian mammals, although relatively few small-bodied species were included in the analysis. Within particular phylogenetic groups, significant negative relationships between body mass and milk protein, dry matter, fat, or energy have been found (Martin 1984, Merchant et al. 1989, Derrickson et al. 1996, Hinde and Milligan 2011) whereas others show no allometric relationships (Oftedal and Jenness 1988, Tilden and Oftedal 1997, Hood et al. 2001). Small species are expected to produce more highly concentrated and higher energy milk because smaller animals have higher mass-specific metabolic demands and reduced digestive capacity due to smaller gastrointestinal tracts (Blaxter 1961). Thus, offspring of small-bodied species should be incapable of ingesting greater quantities of milk to meet metabolic and nutritional demands and should instead require more highly concentrated and energy dense milk (Blaxter 1961).

Arid-adapted. Water balance is a concern for species inhabiting xeric environments (Schmidt-Nielsen and Schmidt-Nielsen 1952); thus the production of highly concentrated milk might be expected in order to reduce maternal water loss through milk. However, some arid-adapted species, such as the camel, *Camelus bactrianus*, and zebras, *Equus burchelli* and *Equus zebra*, produce relatively dilute milk, which is thought to have evolved to facilitate evaporative cooling of offspring (Oftedal and Iverson 1995). Whether an arid-adapted species produces concentrated or dilute milk may depend on body mass. Larger bodied species that require water for evaporative cooling are predicted to have more dilute milk whereas smaller bodied species are expected to produce more concentrated milk because they do not depend on water to dissipate heat (Schmidt-Nielsen and Schmidt-Nielsen 1952). Therefore, we predict an interacting effect of body size and arid adaptation on milk composition.

Flight. Volant mammals are expected to produce concentrated milk for two reasons. First, young must ingest sufficient quantities of protein and minerals to develop the morphological architecture and to attain sufficient body size in order to fly (Hood et al. 2011). Second, to transfer adequate amounts of these vital nutrients, females can either produce small volumes of highly concentrated milk or large volumes of dilute milk. Carrying large volumes of milk would increase wing loading and reduce maneuverability; thus, volant species are expected to produce highly concentrated milk due to their mode of locomotion (Kunz et al. 1995).

Maternal diet. Differences in diet among species may contribute to inter-specific variation in milk composition (Leigh 1994, Kunz et al. 1995, Derrickson et al. 1996) by contributing to differences in the availability of raw materials to the mammary gland for milk synthesis. Mammals consuming primarily herbivorous diets typically produce more dilute milk that is lower in protein and energy than carnivores or omnivores (Jenness and Sloan 1970). This hypothesis is supported in bats where insectivorous bats tend to produce concentrated milk higher in fat and protein than frugivorous bats, suggesting a role of diet in the differences in milk composition seen among species (Kunz and Stern

1995, Messer and ParryJones 1997). Carnivorous species are thought to produce milk higher in fat, protein, and energy because animal matter contains more fat and protein than plants and fruits, which would increase the availability of fatty acids and amino acids that can be incorporated into the milk by the mammary gland (McNab 1980, Morrison 1980, Kunz and Diaz 1995).

Lactation length. Species with longer lactation lengths appear to produce milk lower in gross energy and total solids. For example, the African elephant, *Loxodonta africana*, has an average lactation duration of 3 years (Ernest 2003) and produces milk with 0.9 kcal/g energy and 17% dry matter (Oftedal 1984, Oftedal and Iverson 1995). On the opposite end of the spectrum, the hooded seal, *Cystophora cristata*, which has the shortest lactation length of any mammal at 4 days (Bowen et al. 1985), produces milk with 6 kcal/g energy and 70% dry matter (Oftedal and Iverson 1995). Producing relatively dilute milk over a long lactation period might serve to protect maternal body stores from being depleted prior to completion of neonatal growth and development (Hinde and Milligan 2011). On the other hand, transferring large amounts of nutrients and energy to offspring in a short period of time is thought to reduce maternal maintenance costs during lactation while allowing a greater proportion of energy to be transferred to the young (Fedak and Anderson 1982).

Developmental stage at birth. Martin (1984) proposed that mammals producing precocial young would have more dilute milk than species producing altricial young. This hypothesis was based on the observation that precocial young begin consuming solid food at an earlier age than altricial species and thus would not require as much nutrient transfer from the mother during lactation (Martin 1984). However, there is little evidence in the literature to support this assertion. After controlling for body size, Derrickson et al. (1996) found that among several species of rodents, the precocial species produced milk with a greater fat concentration and energy density than altricial species. Given that precocial young achieve independence earlier and require more energy for thermoregulation than altricial species (Hackländer et al. 2002), we expect precocial species to produce more concentrated and energy dense milk.

Biome (aquatic versus terrestrial). Aquatic mammals typically have more concentrated milk that is higher in fat and energy than terrestrial species (Bonner 1984, Oftedal 1984, Oftedal and Iverson 1995). It has been suggested for pinniped neonates that there is likely a minimum blubber thickness necessary to achieve thermal balance (Drescher 1980) and in fact heat loss occurs at a higher rate in those individuals with low blubber thickness (Worthy 1985). Thus, aquatic animals may produce more concentrated milk that is higher in fat and energy to allow for rapid deposition of an insulating subcutaneous fat layer for neonatal thermoregulation (Jenness and Sloan 1970, Oftedal et al. 1988, Oftedal 1993, Oftedal and Iverson 1995).

Reproductive effort. Lactation is the most energetically expensive phase of mammalian reproduction (Gittleman and Thompson 1988) and higher reproductive output, such as the production of a larger litter mass relative to maternal mass, increases energetic demands and milk energy output during lactation (König et al. 1988). Thus, females with higher reproductive output are expected to produce more concentrated milk with higher fat and protein concentrations and greater energy density because of the greater nutritive and energetic requirements of the litter (Oftedal 1993, Power et al. 2002).

METHODS

Data collation

Species inclusion. We followed the criteria of Oftedal and Iverson (1995) to determine species inclusion in the study. First, milk must have been collected from at least 3 individuals. Second, samples must have been collected by manual palpation from the mammary gland, not from the neonate's stomach or by using vacuum systems. Third, mother and young must not have been separated for an extended period of time (greater than 24 hours) for those species that do not normally have inter-suckling intervals of this length. Fourth, adequate information had to be provided to determine that milk was collected at mid-lactation. Milk composition data for domestic dogs and cats and agricultural species were not included because of the possibility of artificial selection on milk composition.

Delineation of lactation stages. We only included data on milk collected around mid (i.e. peak) lactation following the criteria of Oftedal and Iverson (1995) for delineation of lactation stages. Mid-lactation included the period where milk composition was stable relative to other time points during lactation whereas the initial and final changes in milk composition are considered to be indicative of early and late lactation, respectively. For marsupials, we considered mid-lactation to begin at the plateau in milk composition as young begin emerging from the pouch and ending shortly thereafter. For phocids, where young are weaned before beginning to consume solid foods, we considered mid-lactation to include the entire period extending from when milk composition becomes stable until weaning.

Milk composition. We began by including all milk data contained in Oftedal and Iverson (1995) (n = 100 species) and then conducted a search for articles published between 1995 and 2011 using ISI web of knowledge. From the articles published between 1995 and 2011, 30 additional species met the criteria established above and were included in our study. All milk composition data are expressed on a wet mass basis. Unless indicated otherwise, milk energy (kcal/g) was calculated from the equation $E = 9.11F + 5.86P + 3.95S$, where the units for fat, protein, and sugars are grams per gram of whole milk as in Derrickson et al. (1996). Milk composition data along with analytical techniques employed and references are presented in Appendix 1.

Natural history and ecology. Life history and ecology data were extracted primarily from compendiums and from subsequently published articles when compendiums were missing data. If ranges were provided, the midpoint was calculated. For multiple measures on a single species, averages were obtained and we made sure not to count data from the same source more than once. Developmental stage at birth was based on neonatal independence in four trait categories; thermoregulatory, sensory, locomotory, and nutritionally, following Derrickson (1992). Species were coded with a 1 if the trait was present within 2 days of birth and a 0 if the trait was absent: hair covering body (thermoregulatory), eyes open (sensory), ambulatory without assistance (locomotory), and consumes solid food (nutritionally). Codes were summed across the 4 traits to give a ranking of developmental stage at birth from 0-4, with species receiving a 0 being the most altricial species and those receiving a 4 being the most precocial species. Because of the small sample size of species in the number 4 ranking (n = 2), species ranked as 3 or 4 were combined. Assignment of diet type (carnivorous, omnivorous, or herbivorous) was based on the predominant food type in the diet. Reports of food items occasionally eaten were not included. Arid-adapted species were considered to be those inhabiting xeric regions, typified by low precipitation levels and high temperatures. If the species distribution extended through both mesic and arid regions, the species was considered to be arid-adapted. Reproductive effort was estimated by dividing total litter mass by maternal mass. Total litter mass was calculated by multiplying neonate mass by litter size. Life history and ecology data are presented in Appendix 2.

Phylogeny

We constructed a composite phylogeny (Fig. 1) using the program Mesquite (Maddison and Maddison 2011) based on published phylogenetic trees. Our mammalian tree was taken from Bininda-Emonds (2007), was trimmed to remove species not included in our study, and we added additional species for which we had milk composition data but that were not in the Bininda-Emonds tree (n = 5; *Cervus canadensis nelsoni, Cervus elaphus hispanicus, Equus ferus przewalski, Papio anubis, Papio cynocephalus*). Phylogenetic relationships among *Cervus* species and subspecies were obtained from Randi et al. (2001), *Equus* from Oakenfull et al. (2000), and *Papio* from Newman et al. (2004). Branch lengths were taken from Bininda-Emonds (2007) and for the 5 species added branch lengths were assigned by choosing the arbitrary ultrametricize function in Mesquite. The final tree contained some soft polytomies (n = 6) that were resolved by setting the polytomy branch lengths equal to zero in Mesquite.

Statistical analyses

All variables were log10 transformed prior to running analyses. Statistical analyses were performed using the Matlab (Matlab 2011) regression v2.m program

(Lavin et al. 2008) using different statistical models; ordinary least squares regression (i.e. conventional nonphylogenetic approach; OLS), phylogenetic generalized least squares regression (PGLS), and regression with an Ornstein-Uhlenbeck transformation (RegOU) (Garland et al. 2005). We also used SAS (2002) to confirm output obtained from OLS regressions in Matlab regression v2.m.

The OLS regression assumes no correlation of residuals among species whereas PGLS regression assumes that residuals are correlated among species in a process like Brownian motion character evolution (Lavin et al. 2008) in which branch lengths are proportional to divergence times (Garland et al. 2005). RegOU is based on an Ornstein-Uhlenbeck (OU) process of evolution and is used to model effects of stabilizing selection (Felsenstein 1988, Garland et al. 1993, Blomberg et al. 2003). The OU transformation alters branch lengths to make the tree more or less hierarchical than the original (Blomberg et al. 2003). The regressionv2.m program estimates the optimal OU transformation parameter (*d*) and a value of 1 indicates that the original phylogenetic tree best fits the data whereas a *d* of 0 indicates that a star phylogeny better fits the data. A *d* parameter between 0 and 1 indicates that a tree with branch lengths between the original phylogeny and a star phylogeny best fits the data (Blomberg et al. 2003).

For each milk constituent (e.g. fat, protein, dry matter, and energy), we developed progressively more complex models with the simplest models containing a single independent variable. We then ran models including all possible combinations of the independent variables that were significant from the simplest models that best fit the data. Species with any missing values for the life history/ecology traits were removed from all models for that specific milk constituent so that different statistical models (e.g. OLS, PGLS, and RegOU) for each constituent could be compared. However, models with different dependent variables could not be statistically compared because they include different species and have different sample sizes. For example, for some species we were missing data on dry matter content of milk so these species were removed from all statistical models where dry matter was the dependent variable, but these species were included in all models including the dependent variables fat, protein, and energy (if those data were available).

The fit of the different models to the data were determined using AIC values, where the AIC was calculated in the regression v2.m program by the equation: (-2 x ln ML likelihood) + (2 x number of parameters). Smaller AIC values indicate better fit of that model to the data. Models with AIC values <2 units larger than the best model are also typically considered to have strong support (Burnham and Anderson 2002). Ln maximum likelihood ratio tests (LRTs) were also employed to compare the fit of RegOU to either its PGLS or OLS counterpart for the same model and to compare PGLS and OLS counterparts. The difference in the ln maximum likelihoods between models multiplied by 2 follows a chi-square distribution, with degrees of freedom being equal to the difference in parameters between the two models being compared. PGLS and OLS counterparts, however, have the same number of parameters and thus 0 degrees of freedom. For these comparisons, a difference in twice the ln maximum likelihoods >3.84, which is the 95th percentile of a χ^2 distribution with 1 degree of freedom, indicates a significant difference in the fit of the models to the data (Felsenstein 2004).

LRTs were also used to compare more complex models to simpler models within OLS tests, within PGLS tests and within RegOU tests when models were a nested subset of another (for example the model containing only biome could be compared to biome + diet but not to lactation length + diet). When PGLS or RegOU models are found to better fit the data, based on AIC values or LRTs, than OLS tests, it is indicative of phylogenetic signal, which is the tendency of related species to resemble each other (Grafen 1989, Freckleton et al. 2002, Blomberg et al. 2003). For all statistical tests, $\alpha = 0.05$. When data are presented as averages standard errors are given.

RESULTS

For models with only 1 independent variable (the simplest models), there were no significant relationships between milk composition, including percent dry matter, fat, protein, or energy content, and maternal body mass, reproductive effort, and whether or not a species is volant for all statistical models considered (OLS, PGLS, and RegOU; all $P > 0.05$). Because body mass was not significant we did not include an interaction term between body mass and other independent variables in any models. Biome, lactation length, diet, and arid-adaptation had significant impacts on milk fat concentration; however, arid-adaptation was only significant for the non-phylogenetic OLS model (Table 1). For models of ecological effects on milk protein concentration, biome, developmental stage at birth, lactation length, diet, and number of litters per year were significant, but only diet was still significant when accounting for phylogeny (Table 2). For dry matter, biome, developmental stage at birth, lactation length, diet, and arid-adaptation were significant (Table 3). Only biome, lactation length, and diet were still significant for phylogenetic models, however. Biome, developmental stage at birth, lactation length, and diet had significant impacts on milk energy content, but developmental stage was only significant for the OLS model (Table 4).

Overall, based on AIC values (smaller is better), phylogenetic models (RegOU or PGLS) provided a better fit to the data than their nonphylogenetic counterparts (OLS), indicating strong phylogenetic signal for milk fat, protein, dry matter and energy (Tables 1-4). Furthermore, LRTs showed that both phylogenetic models were statistically significantly better than their nonphylogenetic counterparts. Therefore, a few ecological variables including developmental stage at birth, whether or not a species is arid-adapted, and the number of litters per year, were not included in the more complex models because they were only significant in the OLS statistical models.

For models of fat as the dependent variable, RegOU had the smallest AIC values for all models except for diet and arid-adapted (where the PGLS model had the lowest AIC; Table 1). However, for many models, the difference in AIC values between RegOU and PGLS were <2 units, indicating that both were a strong fit to the data. Based on LRTs, RegOU models were significantly better than their PGLS counterparts only for biome + lactation length ($\chi^2 = 5.84$, *d.f.* = 1, $P = 0.02$) and for lactation length + diet + biome ($\chi^2 = 4.08$, *d.f.* = 1, $P = 0.04$). For diet alone, PGLS was significantly better than RegOU ($\chi^2 = 37.8$, *d.f.* = 1, $P < 0.0001$). Overall, the best model (i.e. the one with the lowest AIC value) was the RegOU model including lactation length + diet followed by the RegOU model of lactation length + diet + biome and then the PGLS model of lactation length + diet (Table 1). The RegOU model of lactation length + diet was not significantly different from the RegOU model of lactation length + diet + biome ($\chi^2 = 0.2$, *d.f.* = 1, $P = 0.66$). This indicates that both models fit the data equally well and that biome does not have a significant impact on milk fat concentration.

For protein, RegOU models had the lowest AIC values, but in some cases the difference in AIC values between PGLS and RegOU was <2 units (Table 2). For all protein models, PGLS was not significantly different from its RegOU counterpart (based on LRTs, all $P > 0.05$). Overall, the best models, based on the lowest AIC values, were the RegOU and PGLS models including diet. More complex models for effects of ecological variables on protein were not developed because out of the simplest models only diet had a significant effect on protein concentration for PGLS and RegOU models.

For dry matter, RegOU models had the lowest AIC values (Table 3) and RegOU models were significantly better than their PGLS counterparts for the following models: lactation length ($\chi^2 = 5.74$, *d.f.* = 1, $P = 0.02$), biome + lactation length ($\chi^2 = 12.54$, *d.f.* = 1, $P = 0.0004$), lactation length + diet ($\chi^2 = 3.88$, *d.f.* = 1, $P = 0.04$) and lactation length + diet + biome ($\chi^2 = 12.12$, *d.f.* = 1, $P = 0.0005$). Overall, the model with the lowest AIC value was the RegOU model including lactation length + diet + biome followed by the RegOU model of lactation length + diet. LRT indicates that the RegOU model of lactation length + diet + biome is significantly better than the RegOU model of lactation length + diet ($\chi^2 = 8.98$, *d.f.* = 1, $P = 0.003$).

For energy, RegOU models had the lowest AIC values (Table 4) and the RegOU models were significantly better than their PGLS counterpart for all models (all $P < 0.02$). The RegOU model of lactation length + diet + biome had the lowest AIC value followed by the RegOU model of lactation length + diet and the models were not significantly different ($\chi^2 = 3.28$, *d.f.* = 1, *P* = 0.07) indicating that biome does not have a significant impact on milk energy density.

Taking the best model for each milk constituent, diet had a significant impact on milk fat, protein, and dry matter concentrations, and energy content (Table 5). Specifically, carnivores had significantly higher concentrations of milk energy content and dry matter, fat, and protein concentrations than omnivores and herbivores (Table 5, Fig. 2). Whether a species is aquatic or terrestrial only had a significant effect on dry matter content of milk (aquatic: 53.6 ± 3.0%, *n* = 21; terrestrial: 21.6 ± 0.9%, *n* = 103; Table 5). A significant negative relationship was found between lactation length and milk fat and dry matter concentrations and energy density (Table 5).

DISCUSSION

Numerous hypotheses have been proposed to explain why milk composition varies among mammalian species but until now it has been unclear to what extent species relatedness and ecology contributes to the observed inter-specific variation in milk composition. Herein, we compiled the most comprehensive data set to date on the gross composition of mammalian milks, including monotremes, marsupials, and placental mammals, and show that for all of the models in our study, statistical models incorporating phylogenetic relatedness (PGLS or RegOU) provided a better fit to the data than conventional non-phylogenetic models (OLS; Tables 1-4). This indicates that some of the similarity in milk composition among closely related species is due to shared common ancestry and thus, milk composition can be said to exhibit a strong phylogenetic signal.

We also compared models using the original phylogenetic tree (PGLS models) to models that apply the branch length transformation; the Ornstein-Uhlenbeck transformation (RegOU). This is recommended because there are errors associated with estimating branch lengths and the model of character evolution is rarely exactly known, particularly for wild populations (Garland et al. 2005); thus, the original tree might not be the best fit to the data that are being mapped on it. Use of alternative trees with branch lengths estimated from different models of character evolution can in some cases provide more statistical power in detecting phylogenetic signal (Garland et al. 1993, Blomberg et al. 2003). In our study, estimates of the optimal OU transformation parameter (*d*) were between 0.6 and 0.9 (Tables 1-4) indicating that for all models the tree that provided a better fit to the data contained a hierarchical structure between a star phylogeny and the original phylogeny used. However, testing the significance of differences between PGLS and RegOU models using likelihood ratio tests showed that RegOU models fit the data significantly better than PGLS for some models whereas for other models RegOU and PGLS both provided a strong fit to the data.

Body mass, reproductive effort, and flight were not correlated to any milk components for the OLS, PGLS, or RegOU models (Tables 1-4). In a comparative study of the milk dry matter content of 62 species of eutherian mammals, Langer (2008) also found no significant relationship between female body mass and percent dry matter both prior to and after accounting for phylogeny. Similarly, no differences in milk composition were found when comparing species within *Pteropus* (Hood et al. 2001), *Eulemur* (Tilden and Oftedal 1997), and *Equus* (Oftedal and Jenness 1988) genera, despite substantial inter-specific variation in body mass. In contrast, other studies have found negative allometric relationships between size and milk dry matter in rodents (Derrickson et al. 1996) and macropod marsupials (Merchant et al. 1989), although allometric relationships on a broader range of taxa should be interpreted with caution when species relatedness is not accounted for as phylogeny may be more influential in driving these trends (Harvey and Pagel 1991). After accounting for phylogeny, Hinde and Milligan (2011) still found a negative relationship between body size and milk energy density in primates. Although body mass might be an important predictor of milk composition among species within certain mammalian groups, it does not appear to have contributed to the evolution of differences in gross milk composition across all mammals. We also expected species capable of flight to produce more concentrated milks as carrying large volumes of more dilute milk would be expected to increase wing loading (Kunz et al. 1995). We found no evidence of an effect of flight on milk composition, however sample size of volant species was relatively low (n = 10 out of 130 species). It would be valuable to re-assess effects of flight on milk composition when data on milk composition become available for more volant species.

Developmental stage at birth and whether a species is adapted to arid environmental conditions each had a significant effect on milk composition for the non-phylogenetic (OLS models), but not for the phylogenetic models (PGLS or RegOU) (Tables 1-4). In the non-phylogenetic model, arid-adapted species were found to produce more dilute milk, which could be interpreted as support for the adaptive significance of evaporative cooling of neonates in desert adapted species. However, lack of significance in the PGLS and RegOU models demonstrates that the evolution of dilute milk is not associated with adaptation to xeric conditions, but rather is accounted for by phylogenetic relatedness and other ecological factors. The different conclusions reached when employing non-phylogenetic and phylogenetic comparative methods underscores the importance in comparative studies of conducting statistical analyses that account for phylogenetic relationships among species.

The best models, based on AIC values and LRTs, indicate that the selective pressures of diet, lactation length, and biome were most influential in the evolution of mammalian milks even after accounting for phylogeny (Tables 1-4). This contrasts Langer (2008) where no relationship was found between diet and milk dry matter. However, Langer (2008) did not categorize species into diet type groups, but instead estimated food quality based on fiber content relative to dry matter content of the main food items ingested and thus did not address the effects of relative variation in protein, fat and energy consumption on milk composition. We found that carnivorous species produce milk higher in fat, protein, and dry matter concentration, and energy content than both herbivorous and omnivorous species (Fig. 2), a pattern that is also observed among frugivorous and insectivorous bats (Messer and ParryJones 1997). Carnivorous species typically ingest higher quantities of fat and protein given that animal matter contains a higher proportion of these nutrients, which is thought to contribute to variation in milk composition among species consuming different diets (McNab 1980, Morrison 1980, Kunz and Diaz 1995).

Lactation length was negatively correlated with milk fat and dry matter concentration and energy density (Table 5) corroborating a phylogenetic comparative analysis of the relationship between lactation length and percent milk fat among pinnipeds (Schulz and Bowen 2005). This lactation strategy likely exists to reduce the cost of milk synthesis and to prevent irreversible damage to maternal somatic tissues. Species that have long lactation lengths would likely be unable to sustain the demands of lactation if they produced highly concentrated and energy dense milk, either due to ceilings on food intake rates or due to substantial self-maintenance costs associated with mobilizing greater quantities of body stores (Hinde and Milligan 2011) and therefore produce dilute milk. On the other hand, species with truncated lactation periods are thought to reduce the costs of lactation by transferring a large quantity of highly concentrated and energy dense milk. In this manner, a greater proportion of maternal energy stores can be turned into gains in pup mass rather than to sustaining maternal somatic maintenance (Fedak and Anderson 1982). Among phocids, a taxa for which detailed information on maternal energy transfer and pup growth during lactation are available, hooded seal (*Cystophora cristata*) mothers lose only 33% of stored fat to increase pup mass by 100% over a 4 day lactation period (Bowen et al. 1987). For species with longer lactation lengths, such as the gray seal (*Halichoerus grypus*), females lose 84% of their stored fat over an average 18 day lactation period and pups have a 236% increase in mass (Fedak and Anderson 1982) and in the northern elephant seal (*Mirounga angustirostris*), females transfer approximately 58% of their fat reserves to increase pup mass by almost 300% during the 26 day lactation period (Costa et al. 1986).

Whether a species occupies an aquatic or terrestrial biome explained some of the variance in milk dry matter concentration (Table 5) with aquatic species producing more concentrated milk. The rate of heat loss in water is much greater than air (Iverson 2002) necessitating the rapid formation of a thick layer of blubber in neonates for thermoregulation (Drescher 1980, Worthy 1985). Therefore, aquatic mammals were expected to produce milk higher in fat concentration and energy density (Jenness and Sloan 1970, Oftedal et al. 1988, Oftedal 1993, Oftedal and Iverson 1995). Interestingly, we found no differences in the concentration of milk fat or protein between aquatic and terrestrial species, suggesting that differences in dry matter content are attributed to differences in total ash (i.e. mineral) or sugar content of milk. In fact, the pinnipeds, which comprise most of the aquatic species in our dataset, produce milk very low in sugar, typically less than 1% of milk wet mass (Oftedal 1984, Oftedal et al. 1987). Although biome had a significant effect on milk fat and energy in the simpler models, those that better fit the data did not include biome indicating that differences of milk fat and protein between aquatic and terrestrial species is explained mainly by differences associated with diet and length of lactation. Alternatively, it is possible that our ability to detect significant differences in milk composition between terrestrial and aquatic species is low due to low sample size for aquatic species (n = 22 out of 130 species).

Although we excluded studies that did not adhere to the guidelines for sample collection, determination of lactation stage, and minimum sample size (see methods) in an attempt to standardize data for comparative purposes, it is important to recognize other potential sources of error among sources from which data were extracted. First, data were collated from studies employing different analytical techniques to determine milk composition. Solvent extraction methods such as the Roese-Gottlieb method for fat, Kjeldahl or CHN techniques for protein, and the phenol-sulfuric acid method for sugars are considered the most reliable techniques for quantifying concentration of milk constituents, whereas others can result in over or under-estimation of milk components (Oftedal and Iverson 1995). Second, species were not raised under identical conditions prior to collecting and analyzing milk, which means that evolutionary differences among species will be to some degree confounded by immediate environmental effects on phenotype (Garland and Adolph 1991, 1994, Garland et al. 2005). However, it is typically unfeasible to maintain animals under identical conditions, particularly when studying wild populations necessitating the assumption that differences among species reflect evolutionary and genetically based differences (Lavin et al. 2008). Third, samples for some species were collected entirely from captive individuals while other studies were conducted on wild populations, which may influence milk synthesis (Munks et al. 1991, Rose and Flowers 2005). Captive species typically have *ad lib* access to food, can achieve larger body sizes, and can rely more heavily on food intake over mobilizing body stores to support milk synthesis, which may alter milk composition. However, other studies have found no differences in milk composition between captive and wild populations of the same species (Messer and ParryJones 1997, Power et al. 2008).

In conclusion, this study exemplifies the need to incorporate phylogenetic relationships among species in comparative studies. Statistical models incorporating phylogenetic relationships among species provide a better fit to the data than conventional non-phylogenetic models. Also, several variables that were significantly correlated to milk composition when a star phylogeny was assumed were not significant when accounting for species relatedness. Most importantly, our results indicate that the evolution of milk consisting of a particular nutritional profile is largely a function of the length of parental care of neonates, maternal dietary intake of nutrients, and whether the species inhabits aquatic or terrestrial biomes. This provides valuable insight into the factors favoring the evolution of one of the key components of mammalian parental care, nutritional provisioning during lactation.

Table 1. Models of the effects of ecological variables on milk fat concentration

Model		ln ML	AIC	*d.f.*	*F*	R^2	*P*	*REML estimate of d*
Biome	OLS	-51.22	108.43	1, 128	78.20	0.38	<0.0001	
	PGLS	-12.11	30.21	1, 128	2.86	0.02	0.09	
	RegOU	-10.49	28.99	1, 128	5.09	0.04	0.03	0.85
Lactation length	OLS	-72.57	151.15	1, 128	20.46	0.14	<0.0001	
	PGLS	-11.42	28.83	1, 128	4.26	0.03	0.04	
	RegOU	-10.32	28.64	1, 128	5.22	0.04	0.02	0.89
Diet	OLS	-50.12	108.25	2, 127	40.53	0.39	<0.0001	
	PGLS	-0.20	8.40	2, 127	14.47	0.19	<0.0001	
	RegOU	-19.10	8.77	2, 127	14.68	0.19	<0.0001	0.90
Arid-adapted	OLS	-78.49	162.98	1, 128	7.54	0.06	0.007	
	PGLS	-12.61	31.22	1, 128	1.85	0.01	0.18	
	RegOU	-10.17	31.61	1, 128	2.21	0.02	0.14	0.91
Lactation length + diet	OLS	-39.90	89.80	3, 126	38.53	0.480	<0.0001	
	PGLS	1.21	7.58	3, 126	10.63	0.200	<0.0001	
	RegOU	2.59	6.83	3, 126	11.36	0.210	<0.0001	0.86
Biome + diet	OLS	-44.99	99.98	3, 126	32.47	0.440	<0.0001	
	PGLS	0.05	9.91	3, 126	9.77	0.190	<0.0001	
	RegOU	1.21	9.59	3, 126	10.28	0.200	<0.0001	0.88
Biome + lactation length	OLS	-37.79	83.59	2, 127	62.24	0.500	<0.0001	
	PGLS	-9.94	27.87	2, 127	3.58	0.050	0.03	
	RegOU	-7.02	24.03	2, 127	7.03	0.100	0.001	0.77
Lactation length + diet + biome	OLS	-32.42	76.84	4, 125	36.01	0.540	<0.0001	
	PGLS	1.49	9.02	4, 125	8.06	0.210	<0.0001	
	RegOU	3.53	6.93	4, 125	9.35	0.230	<0.0001	0.81

Note: OLS = ordinary least squares regression and is a nonphylogenetic model; PGLS and RegOU are models that account for phylogeny, PGLS = phylogenetic generalized least squares regression which is based on a process like Brownian motion; RegOU = regression based on an Ornstein-Uhlenbeck process of evolution; ML = maximum likelihood; *d.f.* = degrees of freedom for the model; *F* = F-statistic for the model; *P* = P-value indicating significance of the model; *d* = OU transformation parameter. Other models tested including maternal body mass, litter mass, and flight, are not presented in the table because they were non-significant for all statistical models considered (OLS, PGLS, and RegOU). Arid-adaptation was not included in the more complex models because it was significant only for the OLS model (which did not fit the data as well as the PGLS and RegOU models).

Table 2. Models of the effects of ecological variables on milk protein concentration

Model		ln ML	AIC	***d.f.***	***F***	***R^2***	***P***	***REML estimate of d***
Biome	OLS	-13.40	32.80	1, 128	18.45	0.13	<0.0001	
	PGLS	56.78	-107.57	1, 128	0.24	0.00	0.63	
	RegOU	58.33	-108.66	1, 128	0.67	0.01	0.41	0.88
Developmental stage at birth	OLS	1.27	7.46	3, 126	18.22	0.30	<0.0001	
	PGLS	57.87	-105.73	3, 126	0.78	0.02	0.51	
	RegOU	60.25	-109.23	3, 126	1.89	0.04	0.13	0.82
Lactation length	OLS	-13.04	32.07	1, 128	19.28	0.13	<0.0001	
	PGLS	56.82	-107.65	1, 128	0.31	0.00	0.58	
	RegOU	58.55	-109.10	1, 128	1.11	0.01	0.29	0.87
Diet	OLS	-10.36	28.72	2, 127	12.63	0.17	<0.0001	
	PGLS	64.03	-120.06	2, 127	7.62	0.11	0.0007	
	RegOU	65.29	-120.58	2, 127	7.57	0.11	0.0008	0.89
Litters per year	OLS	-19.40	44.80	1, 128	5.50	0.04	0.02	
	PGLS	57.67	-109.33	1, 128	1.99	0.02	0.16	
	RegOU	59.33	-110.66	1, 128	2.67	0.02	0.10	0.87

Note: OLS = ordinary least squares regression and is a nonphylogenetic model; PGLS and RegOU are models that account for phylogeny, PGLS = phylogenetic generalized least squares regression which is based on a process like Brownian motion; RegOU = regression based on an Ornstein-Uhlenbeck process of evolution; ML = maximum likelihood; *d.f.* = degrees of freedom for the model; *F* = F-statistic for the model; *P* = P-value indicating significance of the model; *d* = OU transformation parameter. Other models tested including maternal body mass, litter mass, and flight, are not presented in the table because they were non-significant for all statistical models considered (OLS, PGLS, and RegOU). We did not test more complex models as diet was the only variable that was significant in the PGLS and RegOU models (which provided a better fit to the data than their OLS counterparts).

Table 3. Models of the effects of ecological variables on milk dry matter concentration

Model		ln ML	AIC	***d.f.***	***F***	***R^2***	***P***	***REML estimate of d***
Biome	OLS	44.13	-82.25	1, 122	102.13	0.460	<0.0001	
	PGLS	76.24	-146.48	1, 122	4.26	0.030	0.04	
	RegOU	80.21	-152.42	1, 122	9.50	0.070	0.003	0.76
Developmental stage at birth	OLS	10.67	-11.35	3, 120	2.84	0.070	0.04	
	PGLS	74.22	-138.44	3, 120	0.07	0.001	0.98	
	RegOU	75.58	-141.29	3, 120	0.46	0.010	0.71	0.82
Lactation length	OLS	19.63	-33.27	1, 122	28.99	0.190	<0.0001	
	PGLS	80.38	-154.75	1, 122	12.98	0.100	0.0005	
	RegOU	83.25	-158.49	1, 122	15.41	0.110	0.0001	0.81
Diet	OLS	42.17	-76.33	2, 121	47.19	0.440	<0.0001	
	PGLS	86.14	-164.27	2, 121	12.95	0.180	<0.0001	
	RegOU	89.93	-168.24	2, 121	14.50	0.190	<0.0001	0.79
Arid-adapted	OLS	8.48	-10.96	1, 122	4.12	0.030	0.04	
	PGLS	74.11	-142.22	1, 122	0.00	3.0E-05	0.95	
	RegOU	75.92	-143.84	1, 122	0.05	4.0E-05	0.82	0.85
Lactation length + diet	OLS	55.26	-100.53	3, 120	47.92	0.550	<0.0001	
	PGLS	91.27	-172.53	3, 120	12.44	0.240	<0.0001	
	RegOU	93.21	-179.20	3, 120	15.48	0.280	<0.0001	0.73
Biome + diet	OLS	50.37	-90.73	3, 120	41.37	0.027	<0.0001	
	PGLS	86.51	-163.03	3, 120	12.2	0.015	<0.0001	
	RegOU	88.42	-169.09	3, 120	11.36	0.014	<0.0001	0.75
Biome + lactation length	OLS	61.52	-115.05	2, 121	86.81	0.590	<0.0001	
	PGLS	82.39	-156.79	2, 121	8.279	0.130	0.005	
	RegOU	88.66	-167.33	2, 121	14.6	0.210	<0.0001	0.70
Lactation length + diet + biome	OLS	66.36	-120.71	4, 119	48.27	0.620	<0.0001	

	PGLS	91.64	-171.28	4, 119	9.491	0.250	<0.0001	
	RegOU	97.70	-181.39	4, 119	14.28	0.320	<0.0001	0.67

Note: OLS = ordinary least squares regression and is a nonphylogenetic model; PGLS and RegOU are models that account for phylogeny, PGLS = phylogenetic generalized least squares regression which is based on a process like Brownian motion; RegOU = regression based on an Ornstein-Uhlenbeck process of evolution; ML = maximum likelihood; *d.f.* = degrees of freedom for the model; *F* = F-statistic for the model; *P* = P-value indicating significance of the model; *d* = OU transformation parameter. Other models tested including maternal body mass, litter mass, and flight, are not presented in the table because they were non-significant for all statistical models considered (OLS, PGLS, and RegOU). Arid-adaptation and developmental stage at birth were not included in the more complex models because they were significant only for the OLS model (which did not fit the data as well as the PGLS and RegOU models).

Table 4. Models of the effects of ecological variables on the energy content of milk

Model		ln ML	AIC	***d.f.***	***F***	R^2	***P***	***REML estimate of d***
Biome	OLS	12.12	-18.24	1, 112	59.75	0.350	<0.0001	
	PGLS	35.47	-64.93	1, 112	2.67	0.017	0.11	
	RegOU	40.03	-72.06	1, 112	7.98	0.070	0.006	0.73
Developmental stage at birth	OLS	-5.57	21.13	3, 110	4.56	0.110	0.005	
	PGLS	34.74	-59.48	3, 110	0.40	0.010	0.75	
	RegOU	37.62	-63.23	3, 110	0.72	0.020	0.54	0.80
Lactation length	OLS	-0.56	7.11	1, 112	25.51	0.190	<0.0001	
	PGLS	36.33	-66.66	1, 112	4.42	0.040	0.04	
	RegOU	39.96	-71.93	1, 112	7.20	0.060	0.008	0.77
Diet	OLS	16.24	-24.48	2, 111	35.99	0.390	<0.0001	
	PGLS	49.45	-90.90	2, 111	17.12	0.240	<0.0001	
	RegOU	52.63	-95.25	2, 111	18.23	0.250	<0.0001	0.78
Lactation length + diet	OLS	27.37	-44.75	3, 110	36.76	0.500	<0.0001	
	PGLS	50.76	-91.52	3, 110	11.09	0.250	<0.0001	
	RegOU	55.63	-99.26	3, 110	15.28	0.290	<0.0001	0.70
Biome + diet	OLS	20.34	-30.69	3, 110	28.27	0.440	<0.0001	
	PGLS	49.58	-89.16	3, 110	11.3	0.240	<0.0001	
	RegOU	53.45	-94.90	3, 110	13.02	0.260	<0.0001	0.75
Biome + lactation length	OLS	27.36	-46.71	2, 111	55.63	0.500	<0.0001	
	PGLS	37.69	-67.38	2, 111	3.558	0.060	0.03	
	RegOU	45.40	-80.80	2, 111	12.86	0.190	<0.0001	0.60
Lactation length + diet + biome	OLS	33.90	-55.79	4, 109	34.06	0.550	<0.0001	
	PGLS	50.91	-89.81	4, 109	9.267	0.260	<0.0001	
	RegOU	57.27	-100.54	4, 109	13.35	0.330	<0.0001	0.64

Note: OLS = ordinary least squares regression and is a nonphylogenetic model; PGLS and RegOU are models that account for phylogeny, PGLS = phylogenetic generalized least squares regression which is based on a process like Brownian motion; RegOU = regression based on an Ornstein-Uhlenbeck process of evolution; ML = maximum likelihood; *d.f.* = degrees of freedom for the model; *F*–value, *P*-value indicating significance of the model; *d* = OU transformation parameter. Other models tested including maternal body mass, litter mass, and flight, are not presented in the table because they were non-significant for all statistical models considered (OLS, PGLS, and RegOU). Developmental stage at birth was not included in the more complex models because it was significant only for the OLS model (which did not fit the data as well as the PGLS and RegOU models).

Table 5. Significance of ecological variables in full model for milk fat, protein, and dry matter concentration, and energy content

Variable	Coefficient	SE	*F*	*d.f.*	*P*
Fat					
y-intercept	1.01	0.17	33.81		
Lactation length	-0.15	0.08	3.78	1, 126	0.05
Carnivore vs. Herbivore	0.40	0.09	18.39	1, 126	<0.0001
Carnivore vs. Omnivore	0.32	0.06	23.96	1, 126	<0.0001
Herbivore vs. Omnivore	-0.08	0.08	1.02	1, 126	0.28
Diet			13.77	2, 126	<0.0001
Protein					
y-intercept	0.84	0.12	52.91	1, 127	
Carnivore vs. Herbivore	0.19	0.06	10.86	1, 127	0.001
Carnivore vs. Omnivore	0.14	0.04	12.56	1, 127	<0.0001
Herbivore vs. Omnivore	-0.05	0.05	1.04	1, 127	0.31
Diet			7.57	2, 127	0.0008
Dry Matter					
y-intercept	1.44	0.05	834.93		
Lactation length	-0.13	0.04	14.67	1, 119	0.0002
Carnivore vs. Herbivore	0.18	0.04	15.38	1, 119	0.0001
Carnivore vs. Omnivore	0.13	0.03	14.71	1, 119	0.0002
Herbivore vs. Omnivore	-0.04	0.04	1.52	1, 119	0.22
Biome (Aquatic vs. Terrestrial)	0.13	0.06	4.04	1, 119	0.04
Diet			9.30	2, 119	0.0002
Energy					
y-intercept	0.17	0.07	5.39		
Lactation length	-0.12	0.05	6.09	1, 110	0.02

Carnivore vs. Herbivore	0.23	0.05	21.68	1, 110	<0.0001
Carnivore vs. Omnivore	0.30	0.06	23.63	1, 110	<0.0001
Herbivore vs. Omnivore	-0.05	0.05	1.00	1, 110	0.32
Diet			17.45	2, 110	<0.0001

Note: Full models for milk constituents (fat, protein, dry matter and energy content) are all RegOU models which were the best fit to the data based on AIC values (lower is better) and ln maximum likelihood ratio tests (see results). For fat, dry matter, and energy the full model includes lactation length, diet, and biome. For protein, the full model includes only diet. Overall tests for diet are included. The biome variable contained only 2 categories (aquatic and terrestrial) and thus the overall test is the same as the test for aquatic versus terrestrial.

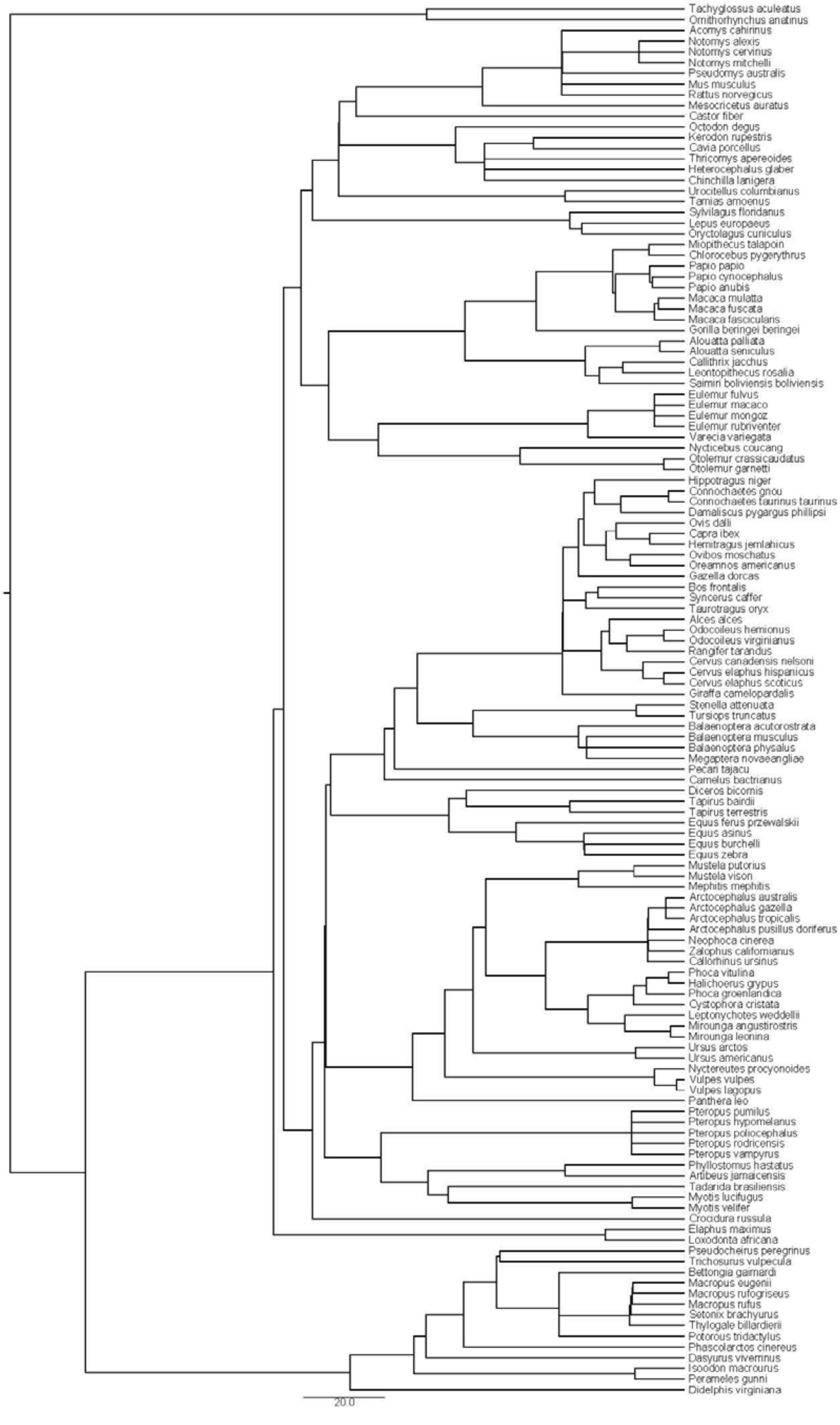
Tachyglossus aculeatus
Ornithorhynchus anatinus
Acomys cahirinus
Notomys alexis
Notomys cervinus
Notomys mitchelli
Pseudomys australis
Mus musculus
Rattus norvegicus
Mesocricetus auratus
Castor fiber
Octodon degus
Kerodon rupestris
Cavia porcellus
Thricomys apereoides
Heterocephalus glaber
Chinchilla lanigera
Urocitellus columbianus
Tamias amoenus
Sylvilagus floridanus
Lepus europaeus
Oryctolagus cuniculus
Miopithecus talapoin
Chlorocebus pygerythrus
Papio papio
Papio cynocephalus
Papio anubis
Macaca mulatta
Macaca fuscata
Macaca fascicularis
Gorilla beringei beringei
Alouatta palliata
Alouatta seniculus
Callithrix jacchus
Leontopithecus rosalia
Saimiri boliviensis boliviensis
Eulemur fulvus
Eulemur macaco
Eulemur mongoz
Eulemur rubriventer
Varecia variegata
Nycticebus coucang
Otolemur crassicaudatus
Otolemur garnetti
Hippotragus niger
Connochaetes gnou
Connochaetes taurinus taurinus
Damaliscus pygargus phillipsi
Ovis dalli
Capra ibex
Hemitragus jemlahicus
Ovibos moschatus
Oreamnos americanus
Gazella dorcas
Bos frontalis
Syncerus caffer
Taurotragus oryx
Alces alces
Odocoileus hemionus
Odocoileus virginianus
Rangifer tarandus
Cervus canadensis nelsoni
Cervus elaphus hispanicus
Cervus elaphus scoticus
Giraffa camelopardalis
Stenella attenuata
Tursiops truncatus
Balaenoptera acutorostrata
Balaenoptera musculus
Balaenoptera physalus
Megaptera novaeangliae
Pecari tajacu
Camelus bactrianus
Diceros bicornis
Tapirus bairdii
Tapirus terrestris
Equus ferus przewalskii
Equus asinus
Equus burchelli
Equus zebra
Mustela putorius
Mustela vison
Mephitis mephitis
Arctocephalus australis
Arctocephalus gazella
Arctocephalus tropicalis
Arctocephalus pusillus doriferus
Neophoca cinerea
Zalophus californianus
Callorhinus ursinus
Phoca vitulina
Halichoerus grypus
Phoca groenlandica
Cystophora cristata
Leptonychotes weddellii
Mirounga angustirostris
Mirounga leonina
Ursus arctos
Ursus americanus
Nyctereutes procyonoides
Vulpes vulpes
Vulpes lagopus
Panthera leo
Pteropus pumilus
Pteropus hypomelanus
Pteropus poliocephalus
Pteropus rodricensis
Pteropus vampyrus
Phyllostomus hastatus
Artibeus jamaicensis
Tadarida brasiliensis
Myotis lucifugus
Myotis velifer
Crocidura russula
Elaphus maximus
Loxodonta africana
Pseudocheirus peregrinus
Trichosurus vulpecula
Bettongia gaimardi
Macropus eugenii
Macropus rufogriseus
Macropus rufus
Setonix brachyurus
Thylogale billardierii
Potorous tridactylus
Phascolarctos cinereus
Dasyurus viverrinus
Isoodon macrourus
Perameles gunni
Didelphis virginiana
20.0

Figure 1. Composite phylogenetic tree used for statistical analyses. The mammalian tree was obtained from Bininda-Emonds et al.(2007) and 5 species (*Cervus canadensis nelsoni, Cervus elaphus hispanicus, Equus ferus przewalski, Papio anubis,* and *Papio cynocephalus)* were added from other published sources. Relationships among *Cervus*, *Equus*, and *Papio* species were taken from Randi et al. (2001), Oakenfull et al. (2000), and Newman et al.(2004), respectively. Branch lengths used are divergence times taken from the Bininda-Emonds et al.(2007) tree and for the 5 species added branch lengths were assigned by choosing the arbitrary ultrametricize function in the program Mesquite. Polytomies were resolved prior to analysis by setting those branch lengths equal to zero.

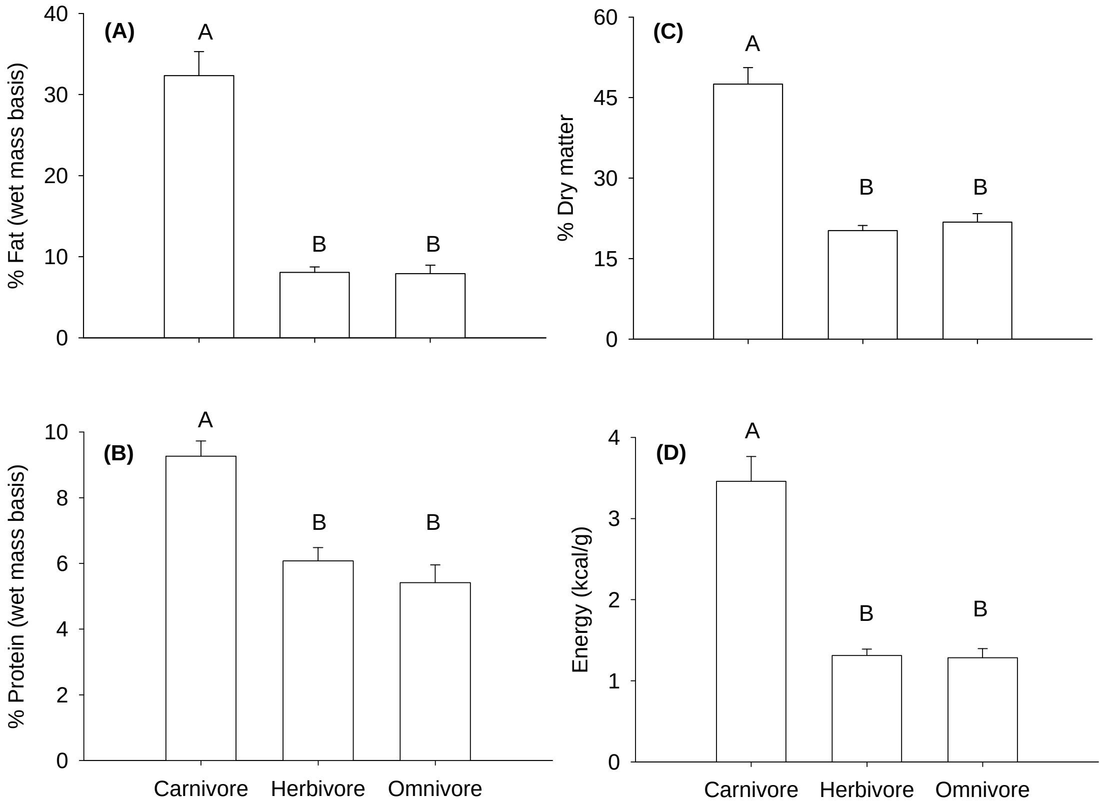

Figure 2. Comparison of milk composition among species with different diets. A) Milk fat concentration, B) Milk protein concentration, C) Milk dry matter concentration, D) Milk energy content. Disparate letters are indicative of statistically significant differences between groups. Error bars are standard errors. Sample sizes for each milk constituent are fat: *n* = 130; protein: *n* = 130; dry matter: *n* = 124; energy: *n* = 114.

Appendix 1. Milk composition of mammals at midlactation

Order	Family	Species	N	Lactation stage	Dry matter (%)	Fat (%)[a]	Protein (%)[b]	Sugar (%)[c]	Energy (kcal/g)[d]	Reference
Artiodactyla	Bovidae	*Bos frontalis*	4+	11-50	20	7.0[V]	6.3[T]	5.2[M]	1.21	Oftedal and Iverson (1995)
Artiodactyla	Bovidae	*Capra ibex*	24	30-60	23.3	12.4[U]	5.7[U]	-	-	Oftedal and Iverson (1995)
Artiodactyla	Bovidae	*Connocheatus taurinus taurinus*	5	150	13.4	7.5[E]	4.1[N]	5.3[H]	1.13	Osthoff et al. (2009a)
Artiodactyla	Bovidae	*Connocheatus gnou*	3	150	12.0	5.5[E]	4.3[N]	4.1[H]	0.91	Osthoff et al. (2009a)
Artiodactyla	Bovidae	*Damaliscus pygargus phillipsi*	4	150	16.0	8.6[E]	5.6[N]	4.9[H]	1.31	Osthoff et al. (2009a)
Artiodactyla	Bovidae	*Gazella dorcas*	16	30-60	24.1	8.8[U]	8.8[U]	-	-	Oftedal and Iverson (1995)
Artiodactyla	Bovidae	*Hemitragus jemlahicus*	9	60?	-	7.9[E]	5.4[N]	3.1[P]	1.16	Oftedal and Iverson (1995)
Artiodactyla	Bovidae	*Hippotragus niger*	6-8	~30-107	17.9	5.0[E,U]	6.2[N]	5.3[P,U]	1.03	Oftedal and Iverson (1995)
Artiodactyla	Bovidae	*Oreamnos americanus*	28	14-35	18	7.0[E]	6.5[N]	4.5[P]	1.20	Oftedal and Iverson (1995)
Artiodactyla	Bovidae	*Ovibus moschatus*	6	~100	28.5	14.3[E]	8.7[N*]	3.6[R]	1.95	Oftedal and Iverson (1995)
Artiodactyla	Bovidae	*Ovis dalli*	4	21-42	22.9	9.5[E]	7.2[N]	5.3[R]	1.50	Oftedal and Iverson (1995)
Artiodactyla	Bovidae	*Syncerus caffer*	5	42-270	19.7	13.4	6.3	5.2	1.80	Osthoff et al. (2009b)
Artiodactyla	Bovidae	*Taurotragus oryx*	11	30-60	21.9	9.9[U]	6.3[U]	4.4[U]	1.44	Oftedal and Iverson (1995)

Artiodactyla	Camelidae	*Camelus bactrianus*	30	23-91	15.2	4.3^{E}	4.3^{N*}	-	-	Oftedal and Iverson (1995)
Artiodactyla	Cervidae	*Alces alces*	15	Mid?	21.5	10.0^{U}	8.4^{U}	3^{U}	1.52	Oftedal and Iverson (1995)
Artiodactyla	Cervidae	*Cervus canadensis nelsoni*[1]	28	14-77	19	6.7^{E}	5.7^{N}	4.2^{P}	1.11	Oftedal and Iverson (1995)
Artiodactyla	Cervidae	*Cervus elaphus*	6	3-31	21.1	8.5^{V}	7.1^{N*}	4.5^{P}	1.37	Oftedal and Iverson (1995)
Artiodactyla	Cervidae	*Cervus elaphus hispanicus*	14	14-26	28.0	12.6^{S}	7.8^{S}	5.8^{S}	1.83	Landete-Castillejos et al. (2000)
Artiodactyla	Cervidae	*Odocoileus hemionus*	24	14-35	18.5	5.5^{E}	7.0^{N}	4.5^{P}	1.09	Oftedal and Iverson (1995)
Artiodactyla	Cervidae	*Odocoileus virginianus*	4+	21-28	22.5	7.7^{U}	8.2^{U}	4.6^{U}	1.36	Oftedal and Iverson (1995)
Artiodactyla	Cervidae	*Rangifer tarandus*	6	21-30	26.3	10.9^{E}	9.5^{N}	3.4^{R}	1.68	Oftedal and Iverson (1995)
Artiodactyla	Giraffidae	*Giraffa camelopardalis*	3	Mid	14.5	4.8^{V}	4.0^{N}	-	-	Oftedal and Iverson (1995)
Artiodactyla	Tayassuidae	*Pecari tajacu*[2]	4	21-48	16.2	4.2^{E}	5.1^{N}	6.2^{M}	0.93	Oftedal and Iverson (1995)
Carnivora	Canidae	*Nyctereutes procyonoides*	22	7-59	18.6	3.4^{E}	7.8^{N}	-	-	Oftedal and Iverson (1995)
Carnivora	Canidae	*Vulpes lagopus*[3]	100?	Mid?	28.6	13.5^{U}	11.1^{U}	3^{U}	2.00	Oftedal and Iverson (1995)
Carnivora	Canidae	*Vulpes vulpes*	3	28-35	18.1	5.8^{E}	6.7^{N}	4.6^{M}	1.10	Oftedal and Iverson (1995)
Carnivora	Felidae	*Panthera leo*	6	45-90	26.8	8.7^{E}	11.8^{N}	3.2^{P}	1.61	Oftedal and Iverson

										(1995)
Carnivora	Mephitidae[4]	*Mephitis mephitis*	15	20-48	30.6	13.8^{E}	9.9^{N*}	3^{P}	1.96	Oftedal and Iverson (1995)
Carnivora	Mustelidae	*Mustela putorius*	18	11-25	-	9.7^{C}	6.9^{L}	3.8^{R}	1.44	Oftedal and Iverson (1995)
Carnivora	Mustelidae	*Neovison vison*[5]	20	10-27	21.7	7.3^{E}	5.6^{N*}	4.5^{P}	1.17	Oftedal and Iverson (1995)
Carnivora	Otariidae	*Arctocephalus australis*	4	~150	54.4	44.4^{E}	9.7^{N}	-	-	Oftedal and Iverson (1995)
Carnivora	Otariidae	*Arctocephalus gazella*	38-66	40-120	55.9	24.4^{O}	5.9^{N}	0.05^{P}	2.57	Arnould and Boyd (1995)
Carnivora	Otariidae	*Arctocephalus tropicalis*	63-78	100-180	66.1	52.1^{E}	12.1^{N}	-	5.69*	Georges et al. (2001)
Carnivora	Otariidae	*Arctocephalus pusillus doriferus*	83	120-315	59.4	47.7^{O}	10.7^{N}	-	-	Arnould and Hindell (1999)
Carnivora	Otariidae	*Callorhinus ursinus*	5	30-120	63.3	50.7^{E}	10.3^{N}	0.1^{R}	5.23	Oftedal and Iverson (1995)
Carnivora	Otariidae	*Neophoca cinerea*	20-38	14-125	37.6	25.4^{E}	10.5^{N}	-	-	Oftedal and Iverson (1995)
Carnivora	Otariidae	*Zalophus californianus*	9	~3-60	41	31.7^{E}	8.6^{N*}	0.3^{P}	3.40	Oftedal and Iverson (1995)
Carnivora	Phocidae	*Cystophora cristata*	15	2-4	69.8	61.1^{E}	4.9^{N}	1.0^{P}	5.89	Oftedal and Iverson (1995)
Carnivora	Phocidae	*Halichoerus grypus*	13	8-15	71.1	59.8^{E}	9.2^{N}	-	-	Oftedal and Iverson (1995)
Carnivora	Phocidae	*Leptonychotes weddellii*	7	10-43	66.2	53.6^{E}	8.9^{N}	0.02^{Z}	5.41	Oftedal and Iverson (1995)
Carnivora	Phocidae	*Mirounga angustirostris*	20-24	20-28	65.8	51.9^{E}	$10.2^{L,N}$	$<0.025^{R}$	-	Oftedal and Iverson (1995)

Carnivora	Phocidae	*Mirounga leonina*	5	11-26	61.5	46.9^{C*}	7.4^{N}	0.02^{A}	4.71	Oftedal and Iverson (1995)
Carnivora	Phocidae	*Pagophilus groenlandica*[6]	8	10-13	65.7	53.5^{E}	7.7^{N}	0.8^{P}	5.36	Oftedal and Iverson (1995)
Carnivora	Phocidae	*Phoca vitulina*	5-15	7-21	62.1	49.9^{E}	9.0^{N}	-	-	Lang et al. (2005)
Carnivora	Ursidae	*Ursus arctos*	9	60-98	31.9	17.1^{E}	9.2^{N*}	2.2^{P}	2.18	Oftedal and Iverson (1995)
Carnivora	Ursidae	*Ursus americanus*	6	60-90	37.6	25.1^{E}	7.0^{N}	3^{P}	2.82	Oftedal and Iverson (1995)
Cetacea	Balaenopteridae	*Balaenoptera acutorostrata*	12	Mid?	41.5	22.2^{U}	14.6^{U}	-	-	Oftedal and Iverson (1995)
Cetacea	Balaenopteridae	*Balaenoptera musculus*	4	~210	55	40.9^{E}	11.9^{N}	1.3^{R}	4.47	Oftedal and Iverson (1995)
Cetacea	Balaenopteridae	*Balaenoptera physalus*	7-9	~210	46.5	33.2^{E}	10.5^{N}	2.3^{R}	3.73	Oftedal and Iverson (1995)
Cetacea	Balaenopteridae	*Megaptera novaeangliae*	8	~300	48.4	33.0^{U}	12.5^{N}	-	-	Oftedal and Iverson (1995)
Cetacea	Delphinidae	*Stenalla attenuata*	3	Mid-late?	-	22.5^{E}	8.4^{N*}	1.2^{R}	2.59	Oftedal and Iverson (1995)
Cetacea	Delphinidae	*Tursiops truncates*	4	198-210	-	29.4^{E}	12.2^{D}	2.5^{P}	3.49	Oftedal and Iverson (1995)
Chiroptera	Molossidae	*Tadarida brasiliensis*	21	22-42	36.5	25.8^{E}	7.7^{N}	3.4^{P}	2.94	Oftedal and Iverson (1995)
Chiroptera	Phyllostomatidae	*Artibeus jamaicensis*	21	13-43	17.8	9.0^{E}	3.6^{N}	6.1^{P}	1.27	Oftedal and Iverson (1995)
Chiroptera	Phyllostomatidae	*Phyllostomus hastatus*	23-32	35-49	25.9	13.1^{E}	9.1^{N}	4.0^{P}	1.88	Stern et al. (1997)
Chiroptera	Pteropodidae	*Pteropus*	7-11	7-159	16.7	6.1^{E}	2.6^{N}	6.4^{P}	0.96	Hood et al.

		pumilus								(2001)
Chiroptera	Pteropodidae	*Pteropus rodricensis*	13-19	7-127	19.1	6.7^{E}	3.4^{N}	6.4^{P}	1.06	Hood et al. (2001)
Chiroptera	Pteropodidae	*Pteropus hypomelanus*	34-43	6-179	18.5	8.5^{E}	2.7^{N}	5.7^{P}	1.16	Hood et al. (2001)
Chiroptera	Pteropodidae	*Pteropus vampyrus*	24-27	4-163	16.5	7.2^{E}	3.1^{N}	5.7^{P}	1.06	Hood et al. (2001)
Chiroptera	Pteropodidae	*Pteropus poliocephalus*	31	51-99	11.2	2.0^{O}	2.5^{D}	6.2^{P}	0.57	Messer and ParryJones (1997)
Chiroptera	Vespertilionidae	*Myotis lucifugus*	3	13-19+	27.1	15.8^{E}	8.5^{N}	4^{P}	2.10	Oftedal and Iverson (1995)
Chiroptera	Vespertilionidae	*Myotis velifer*	3	20-32	25.4	19.9^{E}	10.7^{N}	4.4^{P}	2.61	Oftedal and Iverson (1995)
Dasyuromorphia	Dasyuridae	*Dasyurus viverrinus*	8-35	70-91	29.6	10.9^{E}	7.3^{N}	5.6^{P}	1.64	Oftedal and Iverson (1995)
Didelphimorphia	Didelphidae	*Didelphis virginiana*	3-5	270-330	29.5	11.0^{E}	9.5^{D}	5.9^{P}	1.79	Green et al. (1996)
Diprotodontia	Macropodidae	*Macropus eugenii*	18	168-182	25	4.0^{E}	6.0^{N}	12.5^{P}	1.21	Oftedal and Iverson (1995)
Diprotodontia	Macropodidae	*Macropus rufus*	6	200-232	24.1	6.1^{E}	7.2^{N}	-	-	Oftedal and Iverson (1995)
Diprotodontia	Macropodidae	*Macropus rufogriseus*	8-39	226	25	7.2^{E}	6.8^{N}	10.9^{P}	1.48	Oftedal and Iverson (1995)
Diprotodontia	Macropodidae	*Setonix brachyurus*	3-18	180-270	23.8	10.4^{C}	10.3^{D}	2.5^{P}	1.65	Miller et al. (2009)
Diprotodontia	Macropodidae	*Thylogale billardierii*	71	112-175	10.0	7.1^{C*}	8.0^{D}	8.1^{P}	1.44	Rose and Flowers (2005)
Diprotodontia	Phalangeridae	*Trichosurus vulpecula*	20-23	100-120	24	4.4^{E}	7.0^{D}	11^{P}	1.25	Oftedal and Iverson (1995)
Diprotodontia	Phascolarctidae	*Phascolarctos cinereus*	12	250	31	16.0^{E}	8.0^{D}	4.5^{P}	2.10	Krockenberger (1996)
Diprotodontia	Potoroidae[7]	*Bettongia*	3-6	84-91	25	4.0^{C*}	11.0^{D}	11^{P}	1.44	Oftedal and

		gaimardi								Iverson (1995)
Diprotodontia	Potoroidae[7]	*Potorous tridactylus*	3-5	98-112	27	3.0[C]	10.0[D]	14[P]	1.41	Oftedal and Iverson (1995)
Diprotodontia	Pseudocheiridae[8]	*Pseudocheirus peregrinus*	>8	91-98	23	3.0[C*]	4.5[D]	12.5[P]	1.03	Oftedal and Iverson (1995)
Lagomorpha	Leporidae	*Lepus europaeus*	30	2-26	32.5	15.6[E]	10.0[N]	1.5[Z]	2.07	Oftedal and Iverson (1995)
Lagomorpha	Leporidae	*Oryctolagus cuniculus*	56	5-21	31.2	15.2[C*]	10.3[N]	1.8[M]	2.06	Oftedal and Iverson (1995)
Lagomorpha	Leporidae	*Sylvilagus floridanus*	4	12-15	35.2	14.4[E]	15.8[N]	2.7[M]	2.34	Oftedal and Iverson (1995)
Monotremata	Ornithorhynchidae	*Ornithorhynchus anatinus*	10	Mature	39.1	22.2[E]	8.2[N]	3.7[A]	2.65	Oftedal and Iverson (1995)
Monotremata	Tachyglossidae	*Tachyglossus aculeatus*	15	37-99	48.9	31.0[E]	12.4[N]	2.3[A]	3.64	Oftedal and Iverson (1995)
Peramelemorphia	Paramelidae	*Isoodon macrourus*	8-10	30-37	26	10.0[E]	9.0[N]	6.9[P]	1.71	Oftedal and Iverson (1995)
Peramelemorphia	Paramelidae	*Paremeles gunnii*	7	42-49	45	8.0[E]	9.5[D]	3.3[P]	1.42	Ikonomopoulou et al. (2005)
Perrissodactyla	Equidae	*Equus asinus*	9	30-180	10.8	1.8[E]	1.7[N*]	5.9[P]	0.50	Oftedal and Iverson (1995)
Perrissodactyla	Equidae	*Equus burchelli*	5	90-240	11.3	2.2[E]	1.6[N*]	7.0[P]	0.57	Oftedal and Iverson (1995)
Perrissodactyla	Equidae	*Equus ferus przewalskii*	14	90-360	10.5	1.5[E]	1.6[N*]	6.7[P]	0.50	Oftedal and Iverson (1995)
Perrissodactyla	Equidae	*Equus zebra*	7	90-360	10	1.0[E]	1.6[N*]	6.9[P]	0.46	Oftedal and Iverson (1995)

Perrissodactyla	Rhinocerotidae	*Diceros bicornis*	11	30-330	8.8	0.2^{E}	1.4^{N*}	6.6^{R}	0.36	Oftedal and Iverson (1995)
Perrissodactyla	Tapiridae	*Tapirus bairdii*	4	15-31	13.3	1.9^{E}	4.6^{N}	5.3^{P}	0.65	Oftedal and Iverson (1995)
Perrissodactyla	Tapiridae	*Tapirus terrestris*	3	15-20	15	3.9^{E}	4.4^{N}	5.3^{R}	0.82	Oftedal and Iverson (1995)
Primates	Callitrichidae	*Callithrix jacchus*	10	mid	14.0	3.6^{E}	2.70^{N}	7.4^{P}	0.78	Power et al. (2002)
Primates	Callitrichidae	*Leontopithecus rosalia*	4	10-55	19.4	10.2^{E}	3.0^{N}	6.8^{P}	1.37	Oftedal and Iverson (1995)
Primates	Cebidae	*Aloutta seniculus*	7	30-150	11.3	1.1^{E}	1.9^{N}	6.6^{P}	0.47	Oftedal and Iverson (1995)
Primates	Cebidae	*Aloutta palliata*	7	30-150	11.7	1.6^{E}	2.2^{N}	6.7^{P}	0.54	Oftedal and Iverson (1995)
Primates	Cebidae	*Saimiri boliviensis boliviensis*	8	101-183	16.6	4.6^{E}	3.6^{N}	7.0^{P}	0.91	Milligan et al. (2008)
Primates	Cercopithecidae	*Chlorocebus pygerythrus*	4	90-120	12.9	3.1^{E}	1.6^{N}	8.5^{H}	0.71	Osthoff et al. (2009c)
Primates	Cercopithecidae	*Macaca fascicularis*	8	44-119	12.2	5.2^{E}	1.6^{N*}	-	-	Oftedal and Iverson (1995)
Primates	Cercopithecidae	*Macaca fuscata*	7	35-56	14	4.2^{E}	1.6^{L}	6.2^{Z}	0.72	Oftedal and Iverson (1995)
Primates	Cercopithecidae	*Macaca mulatta*	58	104	-	6.2^{E}	2.1^{N}	7.5^{P}	0.98	Hinde and Milligan (2011)
Primates	Cercopithecidae	*Miopithecus talapoin*	4	17-38	12.3	3.0^{E}	2.1^{N}	7.2^{R}	0.68	Oftedal and Iverson (1995)
Primates	Cercopithecidae	*Papio anubis*	24	21-63	14	4.5^{E}	1.5^{N}	7.8^{R}	0.81	Oftedal and Iverson (1995)
Primates	Cercopithecidae	*Papio*	24	21-63	14	4.5^{E}	1.5^{N}	7.8^{R}	0.81	Oftedal and

		cynocephalus								Iverson (1995)
Primates	Cercopithecidae	*Papio papio*	24	21-63	14	4.5^{E}	1.5^{N}	7.8^{R}	0.81	Oftedal and Iverson (1995)
Primates	Galagidae[9]	*Otolemur garnettii*	14	14-73	18.5	7.3^{E}	5.2^{N*}	6.6^{P}	1.23	Oftedal and Iverson (1995)
Primates	Galagidae[9]	*Otolemur crassicaudatus*	8	19-60	18.6	8.0^{E}	4.8^{N*}	6.4^{P}	1.26	Oftedal and Iverson (1995)
Primates	Hominidae	*Gorilla beringei beringei*	7	30-1500	10.7	1.9^{E}	1.4^{N}	6.8^{P}	0.52	Whittier et al. (2011)
Primates	Lemuridae	*Eulemur fulvus*	6	28-74	9.6	0.9^{E}	1.3^{N*}	8.5^{P}	0.49	Oftedal and Iverson (1995)
Primates	Lemuridae	*Eulemur macaco*	7	30-82	10.1	1.1^{E}	1.5^{N*}	8.4^{P}	0.52	Oftedal and Iverson (1995)
Primates	Lemuridae	*Eulemur rubriventer*	3	26-57	10.3	0.8^{E}	1.1^{N*}	8.9^{P}	0.49	Oftedal and Iverson (1995)
Primates	Lemuridae	*Eulemur mongoz*	4	45-81	9.8	0.7^{E}	1.3^{N*}	7.9^{P}	0.45	Oftedal and Iverson (1995)
Primates	Lemuridae	*Varecia variegata*	5	17-48	14	3.2^{E}	4.2^{N*}	7.7^{P}	0.84	Oftedal and Iverson (1995)
Primates	Lorisidae	*Nycticebus coucang*	4	18-90	16.3	7.0^{E}	3.9^{N*}	6.6^{P}	1.13	Oftedal and Iverson (1995)
Proboscidea	Elephantidae	*Elaphus maximus*	3	60-120	17.7	7.3^{U}	4.5^{N}	5.2^{R}	1.13	Oftedal and Iverson (1995)
Proboscidea	Elephantidae	*Loxodonta africana*	6	60-80	17.3	5.0^{O}	4.0^{N}	5.3^{A}	0.90	Oftedal and Iverson (1995)
Rodentia	Bathyergidae	*Heterocephalus glaber*	3	2-17	17.2	4.50^{E}	4.80^{N}	5.70^{P}	0.92	Hood unpublished
Rodentia	Castoridae	*Castor fiber*	14	10-50	34.1	19.0^{V}	11.2^{N*}	1.7^{M}	2.45	Oftedal and Iverson

										(1995)
Rodentia	Caviidae	*Cavia porcellus*	10	4-9	17.5	5.7^{E}	6.3^{N*}	4.8^{P}	1.08	Oftedal and Iverson (1995)
Rodentia	Caviidae	*Kerodon rupestris*	6	15	20.0	7.5^{E}	6.6^{N}	5.2^{P}	1.28	Derrickson et al. (1996)
Rodentia	Chinchillidae	*Chinchilla lanigera*	60	3-7	20.2	11.2^{E}	7.3^{N}	1.7^{M}	1.52	Oftedal and Iverson (1995)
Rodentia	Cricetidae[10]	*Mesocricetus auratus*	6	Mid	22.6	4.9^{U}	9.4^{N}	4.9^{P}	1.19	Oftedal and Iverson (1995)
Rodentia	Echimyidae	*Thrichomys apereoides*	18-30	7-14	-	22.3^{C}	11.0^{D}	4.4^{R}	2.85	Oftedal and Iverson (1995)
Rodentia	Muridae	*Acomys cahirinus*	5	12	40.5	21.2^{E}	12.3^{N}	2.8^{P}	2.76	Derrickson et al. (1996)
Rodentia	Muridae	*Mus musculus*	5	9-10	40.8	27.0^{C*}	12.5^{N}	2.6^{R}	3.29	Oftedal and Iverson (1995)
Rodentia	Muridae	*Notomys alexis*	3-12	8-14	29.3	15.0^{E}	5.5^{D}	2.6^{R}	1.79	Oftedal and Iverson (1995)
Rodentia	Muridae	*Notomys cervinus*	3-7	8-14	30.2	10.3^{E}	5.6^{D}	2.3^{R}	1.36	Oftedal and Iverson (1995)
Rodentia	Muridae	*Notomys mitchelli*	2-4	8-14	33.3	7.5^{E}	6.5^{D}	2.7^{R}	1.17	Oftedal and Iverson (1995)
Rodentia	Muridae	*Pseudomys australis*	6-7	7-12	25.4	12.1^{E}	6.4^{D}	3.6^{R}	1.62	Oftedal and Iverson (1995)
Rodentia	Muridae	*Rattus norvegicus*	3-18	8-17	22.1	8.8^{E}	8.1^{N*}	3.8^{R}	1.43	Oftedal and Iverson (1995)
Rodentia	Octodontidae	*Octodon degus*	7	15-21	30.5	20.1^{O}	4.4^{D}	2.7^{A}	2.20	Veloso and Kenagy (2005)
Rodentia	Scuiridae	*Tamias amoenus*	11	15-20	36.70	21.7^{O}	8.1^{D}	4.3^{A}	2.62	Veloso et al. (2003)

Rodentia	Sciuridae	*Urocitellus columbianus*	26	19	29.9	9.2^{E}	10.7^{N}	3.4^{P}	1.60	Skibiel and Hood-unpublished
Soricomorpha[11]	Soricidae	*Crocidura russula*	3	8-12	51	30.0^{E}	9.4^{L}	3^{A}	3.40	Oftedal and Iverson (1995)

Note: Table modified from Oftedal and Iverson (1995)

[1]formerly *Cervus elaphus nelsoni*; [2]formerly *Tayassu tajacu*; [3]formerly *Alopex lagopus*; [4]formerly Mustelidae; [5]formerly Mustela vison; [6]formerly *Phoca groenlandica*; [7]formerly Macropodidae; [8]formerly Petauridae; [9]formerly Lorisidae; [10]formerly Muridae; [11]formerly Insectivora

[a]Fat: ^{E}extraction with solvents and gravimetric determination of fat, such as by Rose-Gottlieb and Folch methods (AOAC 1990); ^{V}volumetric measurement of fat after separation of fat in concentrated acid such as Babcock and Gerber methods (see Jenness and Patton 1959); ^{C}measurement of cream layer of capillary tube after centrifugation, such as by methods of Fleet and Linzell (1964) and Ganguli et al. (1969); ^{C*} measurement of cream layer as above but procedure calibrated for species being studied using extraction or volumetric procedures; ^{S}spectrophotometric measurement of lipids such as methods of Stern and Shapiro (1953) and Zöllner and Kirsch (1962); ^{O}other lipid methods; ^{U}uncertain methodology, description of analytical procedures unavailable

[b]Protein: ^{N}total nitrogen multiplied by 6.38, as assayed by the Kjeldahl procedure and various modifications, including the Nessler procedure (Koch and McMeekin 1924) and CHN elemental analysis; ^{N*}protein nitrogen (total nitrogen – non protein

nitrogen) multiplied by 6.38, as assayed by the Kjeldahl procedure and various modifications; ^{D}dye-binding methods, such as procedures using amido black (Weidner and Jakobsen 1966) and Coomassie brilliant blue (Bradford 1976, Sedmark and Grossberg 1977); ^{L}Lowry method (Lowry et al. 1951); ^{T}Biuret method (Gornall et al. 1949); ^{S}spectrophotometric measurement; ^{H}high performance liquid chromatography; ^{U}uncertain methodology, description of analytical procedures unavailable

[c] Sugar: ^{P}phenol-sulfuric acid method (Dubois et al. 1956, Marier and Boulet 1959); ^{R}reducing sugar methods such as copper precipitation method (Munson and Walker 1906), copper titration method (Folin and Wu 1919), picric acid method (Perry and Doan 1950), and chloramine-T method (see Jenness and Patton 1959); ^{A}anthrone method (Morris 1948); ^{Z}enzymatic methods specific for lactose (Bahl 1972); ^{S}spectrophotometric measurement; ^{H}high performance liquid chromatography; ^{M}miscellaneous other methods; ^{U}uncertain methodology, description of analytical procedures unavailable

[d] Energy: All calculated from the formula $E = 9.11F + 5.86P + 3.95L$, where the units for fat, protein, and sugars are grams per gram of whole milk (Derrickson et al. 1996); *determined by bomb calorimetry

Appendix 2. Natural history, life history, and ecology of mammals whose milk composition has been described

Order	Family	Species	Female mass (g)[†]	Lactation length (mths)[‡]	Litter mass (g)[‡]	Repro effort	Developmental stage at birth[*]	Diet	Arid	Flight	Biome
Artiodactyla	Bovidae	*Bos frontalis*	800000[A]	4.5	26949	0.03	3	herbivore	no	no	terrestrial
Artiodactyla	Bovidae	*Capra ibex*	53000	7.5	3489	0.07	3	herbivore	no	no	terrestrial
Artiodactyla	Bovidae	*Connocheatus taurinus taurinus*	170500	8	17717	0.10	3	herbivore	yes	no	terrestrial
Artiodactyla	Bovidae	*Connocheatus gnou*	200000[B]	7.5	11110	0.06	3	herbivore	yes	no	terrestrial
Artiodactyla	Bovidae	*Damaliscus pygargus phillipsi*	61000[C]	4[C]	6500[SS,C]	0.11	3	herbivore	yes	no	terrestrial
Artiodactyla	Bovidae	*Gazella dorcas*	20600[D]	2.8[V]	1771[V]	0.09	3	herbivore	yes	no	terrestrial
Artiodactyla	Bovidae	*Hemitragus jemlahicus*	60000[E]	5	2060	0.03	3	herbivore	no	no	terrestrial
Artiodactyla	Bovidae	*Hippotragus niger*	181000[E]	7	15447	0.09	3	herbivore	yes	no	terrestrial
Artiodactyla	Bovidae	*Oreamnos americanus*	70000	2.7	4193	0.06	3	herbivore	no	no	terrestrial
Artiodactyla	Bovidae	*Ovibus moschatus*	196000	6.2	11188	0.06	3	herbivore	no	no	terrestrial
Artiodactyla	Bovidae	*Ovis dalli*	48400[F]	4.3	4130	0.09	3	herbivore	no	no	terrestrial
Artiodactyla	Bovidae	*Syncerus caffer*	534000	9.2	46292	0.09	3	herbivore	no	no	terrestrial
Artiodactyla	Bovidae	*Taurotragus oryx*	393500[G]	6	28325	0.07	3	herbivore	yes	no	terrestrial
Artiodactyla	Camelidae	*Camelus bactrianus*	585000[H]	10.5	35000	0.06	3	herbivore	yes	no	terrestrial
Artiodactyla	Cervidae	*Alces alces*	269000	3.8	16794	0.06	2	herbivore	no	no	terrestrial
Artiodactyla	Cervidae	*Cervus canadensis nelsoni*[1]	110000[I]	5.5[P]	6500[I]	0.06	2	herbivore	no	no	terrestrial

Artiodactyla	Cervidae	*Cervus elaphus*	98733	12[P]	15500[P,T] T	0.16	2	herbivore	no	no	terrestrial
Artiodactyla	Cervidae	*Cervus elaphus hispanicus*	120333[A]	5.4	9432	0.08	2	herbivore	no	no	terrestrial
Artiodactyla	Cervidae	*Odocoileus hemionus*	43063	2.5	4783	0.11	3	herbivore	no	no	terrestrial
Artiodactyla	Cervidae	*Odocoileus virginianus*	50570	4.3	5394	0.11	3	herbivore	no	no	terrestrial
Artiodactyla	Cervidae	*Rangifer tarandus*	86033	2.8	6928	0.08	3	herbivore	no	no	terrestrial
Artiodactyla	Giraffidae	*Giraffa camelopardalis*	880000	8.3	66943	0.08	3	herbivore	yes	no	terrestrial
Artiodactyla	Tayassuidae	*Pecari tajacu*[2]	21675	1.7	1196	0.06	3	omnivore	yes	no	terrestrial
Carnivora	Canidae	*Nyctereutes procyonoides*	5035	1.7	832	0.17	1	omnivore	no	no	terrestrial
Carnivora	Canidae	*Vulpes lagopus*[3]	3000[J]	1.7	577	0.19	1	carnivore	no	no	terrestrial
Carnivora	Canidae	*Vulpes vulpes*	4244	1.8	439	0.10	1	omnivore	no	no	terrestrial
Carnivora	Felidae	*Panthera leo*	139500	7.2	3995	0.03	1	carnivore	yes	no	terrestrial
Carnivora	Mephitidae[4]	*Mephitis mephitis*	2110	2.1	143	0.07	0	omnivore	no	no	terrestrial
Carnivora	Mustelidae	*Mustela putorius*	720	1.6	71	0.10	0	carnivore	no	no	terrestrial
Carnivora	Mustelidae	*Neovison vison*[5]	770	1.6	41	0.05	0	carnivore	no	no	aquatic
Carnivora	Otariidae	*Arctocephalus australis*	60000	13.5	4250	0.07	2	carnivore	no	no	aquatic
Carnivora	Otariidae	*Arctocephalus gazella*	50000	3.9	5933	0.12	2	carnivore	no	no	aquatic
Carnivora	Otariidae	*Arctocephalus tropicalis*	41400	7.9	4420	0.11	2	carnivore	no	no	aquatic
Carnivora	Otariidae	*Arctocephalus pusillus doriferus*	76400	11.5	5956	0.08	2	carnivore	no	no	aquatic
Carnivora	Otariidae	*Callorhinus ursinus*	34500	3.2	5183	0.15	3	carnivore	no	no	aquatic
Carnivora	Otariidae	*Neophoca*	80000	18.8	7075	0.09	2	carnivore	no	no	aquatic

		cinerea									
Carnivora	Otariidae	*Zalophus californianus*	75000	10.8	6817	0.09	3	carnivore	no	no	aquatic
Carnivora	Phocidae	*Cystophora cristata*	350000	0.3	15389	0.04	3	carnivore	no	no	aquatic
Carnivora	Phocidae	*Halichoerus grypus*	155000	0.6	12820	0.08	3	carnivore	no	no	aquatic
Carnivora	Phocidae	*Leptonychotes weddellii*	369500	1.3	28394[H]	0.08	2	carnivore	no	no	aquatic
Carnivora	Phocidae	*Mirounga angustirostris*	363000	0.9	35250	0.10	2	carnivore	no	no	aquatic
Carnivora	Phocidae	*Mirounga leonina*	556500	0.8	39919	0.07	2	carnivore	no	no	aquatic
Carnivora	Phocidae	*Pagophilus groenlandica*[6]	129000	0.4	8477	0.07	2	carnivore	no	no	aquatic
Carnivora	Phocidae	*Phoca vitulina*	87833	1.1	11084	0.13	3	carnivore	no	no	aquatic
Carnivora	Ursidae	*Ursus arctos*	100000	13.8	1168	0.01	0	omnivore	no	no	terrestrial
Carnivora	Ursidae	*Ursus americanus*	88500	6.4	748	0.01	0	omnivore	no	no	terrestrial
Cetacea	Balaenopteridae	*Balaenoptera acutorostrata*	4705000	5	404000[W]	0.09	3	carnivore	no	no	aquatic
Cetacea	Balaenopteridae	*Balaenoptera musculus*	170000000	7.1	2272500	0.01	3	carnivore	no	no	aquatic
Cetacea	Balaenopteridae	*Balaenoptera physalus*	66800000[A]	6.5	1868500	0.03	3	carnivore	no	no	aquatic
Cetacea	Balaenopteridae	*Megaptera novaeangliae*	32400000	9.1	1338250	0.04	3	carnivore	no	no	aquatic
Cetacea	Delphinidae	*Stenalla attenuata*	57400	18	1000[P]	0.02	3	carnivore	no	no	aquatic
Cetacea	Delphinidae	*Tursiops truncates*	141000	18.9	18920	0.13	3	carnivore	no	no	aquatic
Chiroptera	Molossidae	*Tadarida brasiliensis*	12.07	1.3[X]	3[UU]	0.25	0	carnivore	no	yes	terrestrial
Chiroptera	Phyllostomatidae	*Artibeus jamaicensis*	42	2[Y]	5.9[Y,VV]	0.14	2	herbivore	no	yes	terrestrial
Chiroptera	Phyllostomatidae	*Phyllostomus hastatus*	82	8[Z]	16[WW,H]	0.20	2	omnivore	no	yes	terrestrial

Chiroptera	Pteropodidae	*Pteropus pumilus*	173[K]	2.9[K]	45[H,K]	0.26	2	herbivore	no	yes	terrestrial
Chiroptera	Pteropodidae	*Pteropus rodricensis*	350[E]	2.5[AA]	45[AA,E]	0.13	2	herbivore	no	yes	terrestrial
Chiroptera	Pteropodidae	*Pteropus hypomelanus*	522[L]	1.5[L]	74[L]	0.14	2	herbivore	no	yes	terrestrial
Chiroptera	Pteropodidae	*Pteropus vampyrus*	850[M]	2.5[M]	133[M]	0.16	2	herbivore	no	yes	terrestrial
Chiroptera	Pteropodidae	*Pteropus poliocephalus*	800[N]	5.5[N]	69[N]	0.09	2	herbivore	no	yes	terrestrial
Chiroptera	Vespertilionidae	*Myotis lucifugus*	8	0.8[BB]	3[H]	0.38	1	carnivore	no	yes	terrestrial
Chiroptera	Vespertilionidae	*Myotis velifer*	8	1.5[CC]	3[H]	0.38	1	carnivore	no	yes	terrestrial
Dasyuromorphia	Dasyuridae	*Dasyurus viverrinus*	915	4.5[H]	1[H]	0.001	0	carnivore	no	no	terrestrial
Didelphimorphia	Didelphidae	*Didelphis virginiana*	1906	3.3[DD]	1[DD]	0.0005	0	omnivore	no	no	terrestrial
Diprotodontia	Macropodidae	*Macropus eugenii*	5500	10.5[P]	0.4[H]	0.00007	0	herbivore	yes	no	terrestrial
Diprotodontia	Macropodidae	*Macropus rufus*	26025	10.3[P]	0.8[H]	0.00003	0	herbivore	yes	no	terrestrial
Diprotodontia	Macropodidae	*Macropus rufogriseus*	12425	14.5[EE]	0.6[H,EE]	0.00005	0	herbivore	yes	no	terrestrial
Diprotodontia	Macropodidae	*Setonix brachyurus*	2900	8[FF]	0.4[H]	0.0001	0	herbivore	yes	no	terrestrial
Diprotodontia	Macropodidae	*Thylogale billardierii*	4000	9[GG]	0.4[H]	0.0001	0	herbivore	no	no	terrestrial
Diprotodontia	Phalangeridae	*Trichosurus vulpecula*	2150	6.5[HH]	2[H]	0.0009	0	omnivore	yes	no	terrestrial
Diprotodontia	Phascolarctidae	*Phascolarctos cinereus*	5683	11.4[H]	0.4[H]	0.00007	0	herbivore	no	no	terrestrial
Diprotodontia	Potoroidae[7]	*Bettongia gaimardi*	1725[O]	3.8[FF]	0.3[H]	0.0002	0	herbivore	yes	no	terrestrial
Diprotodontia	Potoroidae[7]	*Potorous tridactylus*	1047	1.4[II]	0.3[H]	0.0003	0	omnivore	yes	no	terrestrial
Diprotodontia	Pseudocheiridae[8]	*Pseudocheirus peregrinus*	910[P]	7.3[JJ]	0.6[H]	0.0007	0	herbivore	no	no	terrestrial
Lagomorpha	Leporidae	*Lepus europaeus*	3961	0.9	337	0.09	3	herbivore	no	no	terrestrial
Lagomorpha	Leporidae	*Oryctolagus*	1444	0.9	219	0.15	0	herbivore	no	no	terrestrial

		cuniculus									
Lagomorpha	Leporidae	*Sylvilagus floridanus*	1237	0.8	176	0.14	1	herbivore	no	no	terrestrial
Monotremata	Ornithorhynchidae	*Ornithorhynchus anatinus*	1268	3.5[KK]	0.6[VV,H]	0.0005	0	carnivore	no	no	aquatic
Monotremata	Tachyglossidae	*Tachyglossus aculeatus*	3600	3[P]	0.3[XX]	0.00008	0	carnivore	yes	no	terrestrial
Peramelemorphia	Paramelidae	*Isoodon macrourus*	877	2.3[LL]	0.7[H,P]	0.0008	0	omnivore	no	no	terrestrial
Peramelemorphia	Paramelidae	*Paremeles gunnii*	736	2.5[MM]	0.5[H]	0.0007	0	omnivore	no	no	terrestrial
Perrissodactyla	Equidae	*Equus asinus*	250000[Q]	13[Q]	29167[NN,E]	0.12	3	herbivore	yes	no	terrestrial
Perrissodactyla	Equidae	*Equus burchellii*	276000	10.8	32292	0.12	3	herbivore	yes	no	terrestrial
Perrissodactyla	Equidae	*Equus ferus przewalskii*	250000	10.5[NN]	30000	0.12	3	herbivore	yes	no	terrestrial
Perrissodactyla	Equidae	*Equus zebra*	262000	10	30000	0.01	3	herbivore	yes	no	terrestrial
Perrissodactyla	Rhinocerotidae	*Diceros bicornis*	884000	19.9	34167	0.04	2	herbivore	yes	no	terrestrial
Perrissodactyla	Tapiridae	*Tapirus bairdii*	300000	12[OO]	9400	0.03	3	herbivore	no	no	terrestrial
Perrissodactyla	Tapiridae	*Tapirus terrestris*	187500	9.5	5418	0.03	3	omnivore	no	no	terrestrial
Primates	Callitrichidae	*Callithrix jacchus*	323	5.4	61	0.19	2	omnivore	no	no	terrestrial
Primates	Callitrichidae	*Leontopithecus rosalia*	570	4.3	107	0.19	2	omnivore	no	no	terrestrial
Primates	Cebidae	*Aloutta seniculus*	5390	12.3	354	0.07	2	herbivore	no	no	terrestrial
Primates	Cebidae	*Aloutta palliata*	6400	14.2	369	0.06	2	herbivore	no	no	terrestrial
Primates	Cebidae	*Saimiri boliviensis boliviensis*	750	5[PP]	107[H]	0.14	2	omnivore	no	no	terrestrial
Primates	Cercopithecidae	*Chlorocebus pygerythrus*	5750[P]	8.5[P]	318[YY,P]	0.06	2	omnivore	no	no	terrestrial
Primates	Cercopithecidae	*Macaca fascicularis*	3233	9.6	408	0.13	2	carnivore	no	no	terrestrial

Primates	Cercopithecidae	*Macaca fuscata*	10750	12.1	672	0.06	2	omnivore	no	no	terrestrial
Primates	Cercopithecidae	*Macaca mulatta*	5140	9.5	476	0.09	2	omnivore	yes	no	terrestrial
Primates	Cercopithecidae	*Miopithecus talapoin*	1120	5.4	188	0.17	2	omnivore	no	no	terrestrial
Primates	Cercopithecidae	*Papio anubis*	12933	7[P]	1068[b,c]	0.08	2	omnivore	no	no	terrestrial
Primates	Cercopithecidae	*Papio cynocephalus*	11725	7 [P]	854[P,c]	0.07	2	omnivore	no	no	terrestrial
Primates	Cercopithecidae	*Papio papio*	19500[P]	7 [P]	1000[d,c]	0.05	2	omnivore	no	no	terrestrial
Primates	Galagidae[9]	*Otolemur garnettii*	731[A]	5[QQ]	49	0.07	3	omnivore	no	no	terrestrial
Primates	Galagidae[9]	*Otolemur crassicaudatus*	935	4.5	80	0.09	3	omnivore	no	no	terrestrial
Primates	Hominidae	*Gorilla beringei beringei*	90000	42[RR]	1600[RR]	0.02	2	herbivore	no	no	terrestrial
Primates	Lemuridae	*Eulemur fulvus*	2500[R]	5.0[R]	85	0.03	2	omnivore	no	no	terrestrial
Primates	Lemuridae	*Eulemur macaco*	2250 [R]	5.2	79	0.04	2	omnivore	no	no	terrestrial
Primates	Lemuridae	*Eulemur rubriventer*	2000 [R]	3.3	86	0.04	2	omnivore	no	no	terrestrial
Primates	Lemuridae	*Eulemur mongoz*	1350 [R]	5.1	71	0.05	2	omnivore	no	no	terrestrial
Primates	Lemuridae	*Varecia variegata*	3750 [R]	3.7	205	0.05	2	herbivore	no	no	terrestrial
Primates	Lorisidae	*Nycticebus coucang*	900	5.8	53	0.06	2	omnivore	no	no	terrestrial
Proboscidea	Elephantidae	*Elaphus maximus*	2720000[S]	18	100039[H]	0.04	3	herbivore	no	no	terrestrial
Proboscidea	Elephantidae	*Loxodonta africana*	2482500	39.4	100986	0.04	3	herbivore	yes	no	terrestrial
Rodentia	Bathyergidae	*Heterocephalus glaber*	80	1.2	17	0.21	0	herbivore	yes	no	terrestrial
Rodentia	Castoridae	*Castor fiber*	19000	2.3	1700	0.09	3	herbivore	no	no	terrestrial
Rodentia	Caviidae	*Cavia porcellus*	728[A]	0.6	366	0.50	4	herbivore	no	no	terrestrial

Rodentia	Caviidae	*Kerodon rupestris*	950[P]	1.1[H]	126	0.13	3	herbivore	yes	no	terrestrial
Rodentia	Chinchillidae	*Chinchilla lanigera*	365[T]	1.8	64	0.18	3	herbivore	yes	no	terrestrial
Rodentia	Cricetidae[10]	*Mesocricetus auratus*	105[A]	0.7	20	0.19	0	omnivore	yes	no	terrestrial
Rodentia	Echimyidae	*Thrichomys apereoides*	374[U]	1.5	66	0.18	4	herbivore	yes	no	terrestrial
Rodentia	Muridae	*Acomys cahirinus*	41	0.8	14	0.34	3	herbivore	yes	no	terrestrial
Rodentia	Muridae	*Mus musculus*	16	0.7	8	0.50	0	omnivore	no	no	terrestrial
Rodentia	Muridae	*Notomys alexis*	31[A]	1.0	11	0.35	1	omnivore	yes	no	terrestrial
Rodentia	Muridae	*Notomys cervinus*	40[A]	1.1	8	0.20	1	omnivore	yes	no	terrestrial
Rodentia	Muridae	*Notomys mitchelli*	50[A]	1	13[e]	0.26	1	omnivore	yes	no	terrestrial
Rodentia	Muridae	*Pseudomys australis*	65 [A]	0.9	13	0.2	0	herbivore	yes	no	terrestrial
Rodentia	Muridae	*Rattus norvegicus*	253	0.8	51	0.20	0	omnivore	no	no	terrestrial
Rodentia	Octodontidae	*Octodon degus*	235 [A]	1.2	74	0.31	3	herbivore	yes	no	terrestrial
Rodentia	Sciuridae	*Tamias amoenus*	53	1.5	14	0.26	0	omnivore	no	no	terrestrial
Rodentia	Sciuridae	*Urocitellus columbianus*	406	1.0	32	0.08	0	herbivore	no	no	terrestrial
Soricomorpha[11]	Soricidae	*Crocidura russula*	14	0.8	4	0.29	0	carnivore	no	no	terrestrial

Notes: Repro effort = reproductive effort calculated as litter mass divided by female mass. Litter mass was calculated as neonate mass multiplied by litter size

Developmental stage ranged from 0-3 with 0 being the most altricial and 3 being the most precocial (see methods)

[1]formerly *Cervus elaphus nelsoni*; [2]formerly *Tayassu tajacu*; [3]formerly *Alopex lagopus*; [4]formerly Mustelidae; [5]formerly Mustela vison; [6]formerly *Phoca groenlandica*; [7]formerly Macropodidae; [8]formerly Petauridae; [9]formerly Lorisidae; [10]formerly Muridae; [11]formerly Insectivora

References: [†]from Silva and Downing (1995) unless indicated otherwise; [‡]from Ernest (2003) unless indicated otherwise; [A]Ernest (2003); [B]Ruckstuhl and Neuhaus (2002); [C]Kingdon (1997); [D]Maltz and Shkolnik (1984); [E]Macdonald (2001); [F]Bowyer and Leslie (1992); [G]Pappas (2002); [H]Hayssen et al. (1993); [I]Grzimek (1975); [J]Murray and Lariviere (2002); [K]Reeder et al. (2006); [L]Jones and Kunz (2000); [M]Kunz and Jones (2000); [N]Menkhorst (1995); [O]Rose and Rose (1998); [P]Nowak (1999); [Q]Grinder et al. (2006); [R]Garbutt (1999); [S]Shoshani and Eisenberg (1982); [T]Spotorno et al. (2004); [U]Redford and Eisenberg (1992); [V]Yom-Tov et al. (1995); [W]Tinker (1988); [X]Nowak (1991); [Y]Merritt (2010); [Z]Willig (1985); [AA]Crichton and Krutzsch (2000); [BB]Anthony and Kunz (1977); [CC]Fitch et al. (1981); [DD]McManus (1974); [EE]Merchant and Calaby (1981); [FF]Tyndale-Biscoe (2005); [GG]Rose (1985); [HH]How (1978); [II]Cronin (2008); [JJ]Thomson and Owen (1964); [KK]Pasitschniak-Arts and Marinelli (1998); [LL]Collins (1973); [MM]Seebeck (2001); [NN]Monfort et al. (1991); [OO]Eisenberg et al. (1990); [PP]Garber and Leigh (1997); [QQ]Smithers (1983); [RR]Taylor and Goldsmith (2003), [SS]Saether and Gordon (1994); [TT]Clutton-Brock et al. (1982); [UU]Whitaker and Hamilton (1998); [VV]Nicol and Andersen (2007); [WW]Kunz and Stern (1998); [XX]Rismiller and McKelvey (2000); [YY]Baldellou and Adan (1997); [ZZ]Green et al. (1997); [a]Hinds et al. (1989); [b]Nicolson (1987); [c]Hearn (1984); [d]Harvey et al. (1987); [e]Jackson (2003)

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CHAPTER TWO

MILK COMPOSITION IN A HIBERNATING RODENT; THE COLUMBIAN GROUND SQUIRREL, *UROCITELLUS COLUMBIANUS*

Abstract. Milk is essential to a mammalian mother's reproductive strategy and is necessary for offspring growth and development. In hibernators with a short duration between weaning and winter immergence, milk synthesis is likely constrained by time and tradeoffs between maternal and offspring condition, thus impacting milk synthesis. We characterized the proximate and mineral composition of milk produced by a hibernating rodent, the Columbian ground squirrel, *Urocitellus columbianus*. The concentration of all milk components varied across lactation; the concentration of most constituents peaked between days 14 and 19 postpartum. Columbian ground squirrel milk was relatively low in lipids but high in protein and calcium. At peak lactation, milk was composed of 10.71 ± 0.46% protein, 9.15 ± 0.47% lipids, 3.39 ± 0.13% sugar, and 0.47 ± 0.02% calcium (wet mass basis). High protein and energy from protein in milk corroborates earlier reports of the importance of fast growth rates of juveniles to over-winter survival whereas the low lipid content of milk may reflect the need for fat conservation for adults. Production of high calcium milk may be a preventive mechanism enabling offspring to cope with bone mineral loss during hibernation.

INTRODUCTION

Lactation is a unique mammalian characteristic that has evolved through numerous physiological and morphological adaptations that support the postpartum production of milk. These adaptations increase the amount of metabolizable nutrients that a lactating mother has available, much of which will be partitioned to milk production (Hammond 1997). Consisting of water, lipids, sugars, and protein (Oftedal 1984), and to a lesser extent, vitamins, minerals and other biologically active compounds, milk is highly digestible (Robbins et al. 1981) and supports young from parturition to independence (Peaker 1977). Because the nutritional building blocks used to produce milk must be obtained, metabolized, and synthesized by the mother, milk production can come at a high metabolic cost to the female (Gittleman and Thompson 1988). Although costly, milk is imperative for offspring growth and survival; thus, milk components are likely under strong selection pressure (Derrickson et al. 1996) and milk production may be one of the best measures of reproductive effort for mammalian mothers (Festa-Bianchet et al. 1994, Stern et al. 1997).

The nutritional composition of milk represents a balance between providing adequate nutrition for offspring while concomitantly minimizing maternal costs associated with milk synthesis (Milligan et al. 2008). The tradeoff between maternal costs of milk production and fitness benefits of producing a nutrient rich milk may be particularly pronounced when a female’s survival is dependent on her body condition at weaning. One clear example of this is in species that enter hibernation shortly after the reproductive period has ended. Unfortunately, knowledge about the composition of milks produced by hibernating species is virtually nonexistent.

Hibernators that do not cache food over-winter rely almost entirely on endogenous adipose stores to support their metabolism over-winter (Dark 2005). Preparation for this change in physiological state requires a period of hyperphagia associated with increases in food intake rates and deposition of body fat (Kenagy 1987, Kenagy and Barnes 1988, Dark 2005). In many species, hibernation typically begins several months after the young are weaned and thus females and juveniles may have adequate time available for foraging to enhance condition prior to hibernation. For example, female bats in temperate regions typically wean pups by late summer and enter hibernation by late autumn or early winter (Racey and Entwistle 2000). In contrast, Columbian ground squirrels begin hibernation just weeks after weaning occurs (Young 1990, Dobson et al. 1992). Thus, an interaction between milk composition and hibernation strategy may be strong in this species.

The purpose of this paper is to characterize milk composition in a hibernating rodent, the Columbian ground squirrel. This species does not cache food in the hibernacula and relies on fat catabolism as the primary energy source during hibernation (Murie and Boag 1984, Young 1990, Dobson et al. 1992). Female squirrels nurse pups for approximately 27 days, during this time the only source of nutrition for pups is through the mother's milk (Murie 1992), and pups are weaned just a few weeks before hibernation begins (Young 1990, Dobson et al. 1992). Thus, pups have little time to forage independently and gain fat mass prior to hibernation. Females that wean heavier juveniles with faster growth rates during lactation have a greater number of young survive their first hibernation (Skibiel et al. 2009) indicating that females can alter offspring survival rates via her lactation behavior or the milk she produces. Because the deposition of adipose tissue and fast growth prior to hibernation is essential for juvenile over-winter survival, the production of high fat milk should be especially important for this species. High protein concentrations in the milk are also expected which would facilitate faster growth rates. In addition, many small hibernating mammals lose bone mass during hibernation (McGee-Lawrence et al. 2008) and thus we expect milk minerals associated with skeletal growth (particularly calcium) to be high providing a potential preventative mechanism for bone loss during hibernation.

We also describe changes in milk composition over the course of the lactation period. In most mammals studied to date, concentrations of milk constituents change throughout lactation, however, the direction and pattern of change varies from species to species (Oftedal 1984, Oftedal and Iverson 1995). Typically fat and protein increase while sugar content decreases; however, there are many exceptions to this general trend (Oftedal 1984). For example, in the house mouse, *Mus musculus*, milk fat concentration increases whereas there is no change in protein throughout lactation (Knight et al. 1986). In the yellow pine chipmunk, *Tamias amoenus*, milk fat and protein content increase whereas sugar content does not vary much from early to late lactation (Veloso 2003). Because fat and protein and minerals associated with skeletal development are likely to be important to Columbian ground squirrel pups, we expect the concentrations of these components to increase throughout lactation.

METHODS

Field and laboratory methods

A population of Columbian ground squirrels was studied on Meadow Dot (N 50°38'59.6" W 114°39'40.9", elevation 1565 m) in the Sheep River Provincial Park in Alberta, Canada in 2009. All animals were captured within 2 days following spring emergence from hibernation using Tomahawk Live Traps (Tomahawk #201 collapsible chipmunk trap, 48 x 15 x 15 cm, Tomahawk, Wisconsin, USA) baited with peanut butter. Animals were given uniquely numbered fingerling eartags and, to facilitate observation from a distance, the dorsal pelage of each individual was painted with a unique dye marking (Lady Clairol Hydrience #51 Black Pearl; Proctor and Gamble, Stamford, CT, USA). Mating dates of females were determined by examining reproductive morphology and by observations of mating behavior.

All females that mated were captured 2-3 days before the estimated parturition date (24 day average gestation; Shaw 1925, Murie and Harris 1982) and transported to the field laboratory to give birth. Following this method, the exact date of parturition was known for all individuals. Females were maintained in individual polycarbonate microvent rat cages (267 x 483 x 20 mm^3; Allentown Caging Equipment Company, Allentown, New Jersey, USA) filled with pine chip bedding and strips of newspaper for nesting material and covered in vented black plastic bags. Squirrels were fed a diet of show-horse feed (EQuisine; oats, barley, wheat, and compressed vegetable material in a molasses mix) given *ad libitum* and lettuce and apple twice daily. Cages were monitored for pups 3-4 times per day between 0700 and 2200 hours. Mother and pups were released back on the field 1-2 days following parturition by opening the cage and allowing the mother to retrieve her pups and take them to her nest burrow.

Milk was collected from reproductive females (*n* = 36) on day 4, 9, 14, 19, and 25 postpartum (day of birth = day 0). Eight of these females lost their litter during the lactation period, leaving 28 females for which we have milk samples from all 5 collection days. Females were captured in the field and held in traps for 3 hours before being milked to allow for milk accumulation within the mammary glands. Animals were anesthetized by placing them in a 4 L plastic container with a screw top lid containing cottonball pre-soaked in isoflurane. Oxytocin (Osborn, Bimeda Inc, Oakbrook Terrace, IL, concentration = 20 USP per ml, dose = 0.5 ul/g) was administered intramuscularly to stimulate milk let down. Hair surrounding the nipple was trimmed and the nipple and surrounding area were cleaned with isopropyl alcohol prior to milk expression. Milk was expressed by light manual palpation of the mammary gland directly into a glass capillary tube, immediately transferred to screw top storage vials, and stored on ice until transported to the field station. Samples were then stored at -20°C until they were shipped on dry ice to Auburn University and subsequently stored at -80°C until assaying. All methods used in this study follow ASM guidelines and were approved by the Auburn University Institutional Animal Care and Use Committee, protocol number 2009-1151 and the University of Calgary Life and Environmental Sciences Animal Care Committee, protocol BIO 9R-42.

Milk analysis

Proximate and mineral composition of milk followed methods for small quantities of milk (Hood et al. 2009). Dry matter content of milk was determined based on change in mass following drying at 100°C for 3 hours in a forced convection oven (Binder drying oven FED 115-UL, Binder Inc., Great River, NY). The crude protein content of milk was determined based on CHN elemental analysis (Perkin Elmer PE2400 Series II CHNS/O Elemental Analyzer, Shelton, CT). Crude protein was estimated by multiplying total nitrogen in the sample by the standard conversion factor, 6.38 (Jones 1931). Total lipid content was estimated by the Roese-Gottlieb ether extraction method modified for micro volumes of milk (AOAC 1990) and sugar content was measured through the phenol-sulfuric acid method (Dubois et al. 1956, Marier and Boulet 1959). The resulting sugar values were multiplied by 0.95 to correct for hydration of the lactose monohydrate standard used in the assay.

For determination of mineral composition, wet samples were first digested in trace metal grade nitric acid in a Speedwave MWS-2 microwave digester (BERGHOF Products + Instruments GmbH, Eningen, Germany) with the microwave ramped to 200°C over 15 min, held at 200°C for 15 min, and then ramped back down to room temperature over the final 15 min. Samples were diluted with plasma grade water (Fisher Chemical, Fair Lawn, NJ) and the mineral content (calcium, sodium, potassium, and magnesium) was then determined by inductively coupled plasma optical emission spectrometry (Perkin Elmer Optima 7300DV; Waltham, MA). An internal standard (Ag) was added to all samples prior to ICP analysis to determine recovery of minerals in the samples. Samples were assayed in duplicate or triplicate as allowed by sample volume and concentrations of milk constituents were averaged across replicates. Mineral replicates with internal standard recoveries less than 90% were excluded from averages for that sample. All assays were validated using either whole cow's milk (sugar and lipid assays) or powdered milk from the National Institute of Standards and Technology (NIST; dry matter, protein, and mineral assays). Recoveries for cow's milk were: sugars = 98% (n = 40), lipids = 92% (n = 42). Recoveries for NIST milk powder are as follows (n = 9 for all assays): dry matter = 98%, protein = 95%, Ca = 111%, K = 81%, Mg = 102%, Na = 103%. Thus, estimates of lipid and K concentrations in squirrel milk may be lower than the actual concentration and estimates of Ca may be slightly high. Gross energy (GE) content of milk was estimated from energy equivalents of protein, sugars, and fat by the equation $E = 9.11F + 5.86P + 3.95S$, where the units for fat, protein, and sugars are grams per gram of whole milk as in Derrickson et al. (1996).

Statistics

All statistical analyses were conducted in SAS (SAS 2002). Normality of data was visualized graphically and determined statistically through Shapiro-Wilks tests. All data were normally distributed with the exception of milk calcium (Ca) and potassium (K) concentrations with Ca having a slight left skew and K having a slight right skew. Transformations did not improve normality so analyses were performed with untransformed data. Separate mixed models (PROC MIXED) were used to test effects of lactation stage on each milk constituent. Female ID was included as a random effect. Day of sample collection was included as a predictor variable as well as day squared to account for potential curvilinear relationships. Because milk composition may vary with litter size (Fiorotto et al. 1991), we included litter size at birth in the models. Litter size did not have a significant effect on any milk component ($P > 0.1$ for all models) and thus, litter size was excluded from all models examining changes in milk composition over time. A Bonferroni correction was applied to adjust the significance level of each model for multiple comparisons. We ran 12 analyses on different milk constituents therefore the α-level was adjusted to 0.004 which was calculated by dividing the α-level of 0.05 by the number of comparisons. Data are presented as means ± SE.

RESULTS

At peak lactation (day 19 postpartum), the dry matter content of Columbian ground squirrel milk was 29.9 ± 0.97% of wet mass. Protein and lipids were the largest proximate constituents in the milk at 10.7 ± 0.46% and 9.15 ± 0.47%, respectively. Milk consisted of 3.39 ± 0.13% sugars. Of the mineral components, calcium occurred in higher concentrations than potassium, sodium, and magnesium (calcium: 0.47 ± 0.02%, potassium: 0.14 ± 0.006%, sodium: 0.09 ± 0.003%, magnesium: 0.02 ± 0.001%). Milk energy at peak lactation was 1.60 kcal/g ± 0.05 kcal/g. Percent of milk energy derived from protein, lipids, and sugars were 39.63 ± 1.53%, 51.61 ± 1.51%, and 8.76 ± 0.54%, respectively.

The concentration of most milk components varied over the course of the lactation period (Table 6) and many constituents showed a distinct peak between 14 and 19 days postpartum (Fig. 3). Of the components assayed, only concentrations of lipids, sugar and sodium, and the proportion of energy from lipids were constant over time (Table 6). Dry matter, protein and energy exhibited similar patterns of change throughout lactation. Dry matter increased from 24% of wet mass to approximately 30% at 19 days postpartum followed by a slight decrease to 27% in late lactation (Fig. 3a) and protein rose from 7% wet mass to 11% and then decreased to 10% by 25 days postpartum (Fig. 3b). Gross energy of milk increased from 1.3 kcal/g to 1.6 kcal/g at 19 days postpartum and then decreased to 1.5 kcal/g at late lactation (Figure 3d). The proportion of milk energy from sugars decreased until peak lactation and then rose to late lactation whereas the proportion of energy from protein showed the opposite pattern of change (Table 6, Fig. 4). Composition of milk calcium changed most dramatically with calcium increasing by 134% from early to peak lactation and then decreasing by 8% by 25 days postpartum (Fig. 3c).

DISCUSSION

Reproduction is constrained by time and energy requirements in hibernating mammals (Heaney 1984) and thus is likely to dictate maternal investment in reproduction particularly for species in which females and juveniles have a short amount of time post-weaning to accumulate fat stores prior to hibernating (Phillips 1984). In Columbian ground squirrels, females and juveniles only have a few weeks post-weaning to increase condition (Young 1990, Dobson et al. 1992) and juveniles that are weaned heavier and grow faster during the lactation period are more likely to survive their first hibernation (Skibiel et al. 2009). For this reason we expected females to produce milk high in fat, protein and energy, which would result in heavier and larger young at weaning.

Although there is insufficient data to apply statistical tests to comparisons of milk produced by our study species to other rodents and hibernating mammals, qualitative comparisons suggest that dry matter, sugar concentration and energy content of Columbian ground squirrel milk at mid lactation (19 days postpartum) are comparable to reports for other rodents and hibernating mammals (Table 7). Although lipids and protein comprised the greatest proportion of wet mass at approximately 9% each, lipid concentration in Columbian ground squirrel milk at mid lactation is relatively low compared to other rodents. For example, house mice and deer mice (*Peromyscus maniculatus*) have 27% and 21% lipids, respectively, at mid-lactation (Oftedal and Iverson 1995, Derrickson et al. 1996; Table 7). Lipid concentration in Columbian ground squirrel milk is up to 20% lower than in hibernating vespertilionid bats (Kunz et al. 1995) and is approximately 13% lower in lipids than that of the yellow-pine chipmunk (*Tamias amoenus*), another hibernating rodent (Veloso et al. 2003; Table 7). In addition, the proportion of energy from lipids was relatively low. This is surprising considering that Columbian ground squirrels do not feed during the hibernation period and thus rely solely on endogenous fat reserves to meet energy needs (Murie and Boag 1984, Young 1990, Dobson et al. 1992). Although yellow-pine chipmunks increase fat mass prior to hibernation (Geiser and Kenagy 1987), they also store food for use during winter (Schulte-Hostedde et al. 2001) and thus may not face the same demands for mass gain as Columbian ground squirrels.

The relatively low lipid concentration found in Columbian ground squirrel milk relative to other hibernators may be attributed to several factors. First, it may be indicative of the importance of fat conservation for adult females in this species. Second, it may reflect the ability of juveniles to compensate for low-fat milk through food consumption post-weaning. Juvenile Columbian ground squirrels typically enter hibernation by the beginning of September whereas adult females enter their first torpor in mid August (Young 1990). This two-week difference in torpor entry by juveniles may afford them a sufficient amount of time to gain fat reserves prior to hibernating. Third, fatty acid composition of milk lipids may be more important for juvenile hibernators than total amount of fat consumed. Unsaturated fatty acids, particularly the essential unsaturated fatty acids such as linolenic and linoleic acids, appear to be especially important for hibernators as sciurids fed diets high in these fatty acids had longer torpor bouts and lower body temperatures during hibernation resulting in substantial energy savings (Frank 1992, Florant et al. 1993). Fourth, the low lipid concentration of Columbian ground squirrel milk may be related to body size. Small mammals, such as shrews (range from 3-18 grams; Nowak 1999) are limited in their ability to store fat to support lactation due to constraints on body size (Oftedal 2000). Likewise, body size is inversely correlated with the concentration of milk dry matter and fat (Derrickson et al. 1996) suggesting that there are also constraints on the volume of milk that small mammals can carry. In bats, more concentrated milk may have evolved to reduce maternal mammary mass and wing-loading, allowing for flight (Kunz et al. 1995). Similarly, for small non-volant mammals, highly concentrated milk reduces milk volume which allows them to maintain agility, whereas larger species, such as Columbian ground squirrels (average 500 grams at peak lactation) should not be under the same constraints to reduce volume of milk by producing highly concentrated and high fat milk.

Protein concentrations in Columbian ground squirrel milk at mid lactation were similar to other hibernating species and to other rodents, but the proportion of milk energy derived from protein was much higher in Columbian ground squirrels (Table 7). Hibernating vespertilionid bats have between 8.5%-10.5% protein (Kunz et al. 1995; Table 7) and Columbian ground squirrel milk consists of approximately 11% protein at mid-lactation. Rodents such as the Norway rat (*Rattus norvegicus*), house mouse, deer mouse, and yellow-pine chipmunk have protein concentrations at mid-lactation between

8% and 12.5% (Oftedal and Iverson 1995, Derrickson et al. 1996, Veloso et al. 2003; Table 7). Small rodents such as these produce young with high growth rates (Case 1978, Reiss 1989) and thus are expected to produce milk higher in protein concentrations than species such as primates that have slow growth rates (primates range from 1.1 to 5.2% protein at mid-lactation; Oftedal and Iverson 1995). In addition, a higher contribution of protein to gross energy is expected in species with faster growth rates. The proportion of milk gross energy from protein in slow-growing primates ranges from 6% to 28% (Power et al. 2002, Hinde et al. 2009) whereas in rodents ranges from ~18% to 40% (Table 7). It is interesting that percent gross energy from protein is much higher in Columbian ground squirrel milk than other species with fast growth rates, suggesting that in this species fast growth is especially important. This corroborates the previous finding of Skibiel et al. (2009) that the number of juveniles surviving hibernation is dependent on growth rate during the lactation period.

Minerals are essential for many physiological and developmental processes. Minerals such as sodium and potassium are involved in osmotic and acid-base balance and calcium, phosphorus, and magnesium are necessary for bone ossification (Brody 1999, Barboza et al. 2009). Calcium concentrations were slightly higher in Columbian ground squirrel milk than in other mammals in general (Studier and Kunz 1995) and other rodents in particular (Table 8). Some empirical evidence suggests that hibernating species, such as ground squirrels, bats and hamsters, lose bone during hibernation due to an increase in bone resorption and a decrease in bone formation (Haller and Zimny 1977, Steinberg et al. 1986, Kwiecinski et al. 1987). However, more recent research on golden-mantled ground squirrels (*Callospermophilus lateralis*) and thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) found no effect of hibernation on geometrical or mechanical properties of the tibia and femur (Utz et al. 2009, McGee-Lawrence et al. 2011). However, there were microstructural differences between hibernating and active thirteen-lined ground squirrels in both cortical and trabecular bone indicating that this species, and potentially other small hibernating mammals, are not completely able to prevent bone loss from disuse during hibernation (McGee-Lawrence et al. 2011). It is possible that elevated calcium concentrations in milk may serve to bolster juveniles against subsequent changes in calcium homoestasis during hibernation. This may be particularly important for Columbian ground squirrels because they do not attain adult structural size until approximately 2 years of age. In addition, because calcium is imperative for bone growth (Barboza et al. 2009; Brody 1999), high amounts of calcium transferred in milk likely also supports rapid structural growth of neonates.

For all mammals whose milk composition has been analyzed, proximate and/or mineral concentration of milk changes as lactation progresses, but the components that change and the pattern of change varies among species (Oftedal 1984, Oftedal and Iverson 1995). Like other rodents, most proximate and mineral components of Columbian ground squirrel milk changed throughout lactation, however the pattern of change differed from other species. In Columbian ground squirrel milk, protein, dry matter, calcium, phosphorus, and magnesium concentrations increased towards peak lactation and then decreased from peak to late lactation rather than increasing linearly from early to late lactation (Fig. 3). Lipids varied little throughout lactation, which quite possibly reflects the importance of lipid acquisition in this hibernating species. As for energy, in many chiropterans and the yellow-pine chipmunk, energy increased from early to late lactation primarily due to an increase in fat (Kunz et al. 1995, Stern et al. 1997, Veloso et al. 2003). Gross energy content of Columbian ground squirrel milk increased to mid lactation and then decreased towards late lactation reflecting the changes in the proportions of milk energy from protein and sugars (Fig. 4).

In conclusion, dry matter and lactose concentration in Columbian ground squirrel milk was similar to bats and rodents whereas lipid and energy content was lower than expected based on the hibernation strategy of this species. It is possible that mothers prioritize the use of fatty acids for maintaining their own adipose stores over using fatty acids and the building blocks of triacylglycerols for milk synthesis. This could be particularly important given that mothers have relatively less time between weaning and hibernation than do their young. Alternatively, the low lipid concentration of Columbian ground squirrel milk may be attributed to their size; rapid fat deposition in the days prior to entering hibernation may make it unnecessary for females or their young to have substantial adipose stores at weaning. We also found that protein and the proportion of milk energy from protein were higher in this species than other rodents and bats indicating the importance of fast growth rates in this species. Finally calcium concentration of Columbian ground squirrel milk was relatively high, potentially contributing to rapid neonatal growth and/or bolstering juveniles against subsequent bone loss during hibernation.

Table 6. Results of mixed models for characterizing changes in the proximate and macromineral concentration and energy content of Columbian ground squirrel milk from parturition to weaning

Milk constituent	predictor	*d.f.*	*F*-value	*P*-value
Proximate				
Dry matter	day	1,113	34.95	<0.0001*
	day^2	1,113	27.63	<0.0001*
Lipids	day	1,111	5.84	0.02
	day^2	1,111	7.13	0.009
Protein	day	1,113	75.79	<0.0001*
	day^2	1,113	49.39	<0.0001*
Sugar	day	1,113	0.31	0.58
	day^2	1,113	5.06	0.03
Minerals				
Calcium	day	1,110	106.72	<0.0001*
	day^2	1,110	65.21	<0.0001*
Magnesium	day	1,111	44.96	<0.0001*
	day^2	1,111	36.09	<0.0001*
Potassium	day	1,111	21.95	<0.0001*
	day^2	1,111	20.55	<0.0001*
Sodium	day	1,111	7.02	0.009
	day^2	1,111	0.93	0.34
Energy				

Gross energy	day	1,110	33.59	<0.0001*
	day^2	1,110	26.44	<0.0001*
GE protein	day	1,110	20.81	<0.0001*
	day^2	1,110	10.88	0.001*
GE lipids	day	1,110	8.40	0.005
	day^2	1,110	2.18	0.14
GE sugars	day	1,110	10.79	0.001*
	day^2	1,110	16.12	0.0001*

Note. The proportion of gross energy (GE) from proximate components are given as GE protein, lipids, and sugars.* indicates significance at an α-level of 0.004 (see materials and methods). Day refers to day of sample collection and day squared was also included in statistical analyses to account for potential curvilinear relationships.

Table 7. Comparison of the proximate composition of milk (%) and percent energy from proximate constituents (in parentheses) at mid lactation among several rodents and hibernating mammals

Taxon	Species	Hiber-nates?	Dry Matter	Sugar	Lipids	Protein	Energy (kJ/g)	Energy (kcal/g)	Ref*
Rodentia, Sciuridae	*Urocitellus columbianus*	Yes	29.9	3.4 (8.6)	9.2 (53.4)	10.7 (40.2)	6.5	1.6	1
	Tamias amoenus	Yes	36.7	4.8 (6.7)	23.4 (75.5)	8.5 (17.6)	11.8	2.8	5
Rodentia, Cricetidae	*Peromyscus maniculatus*	No	32.0	2.4 (3.7)	21. 0 (74.1)	9.9 (22.5)	10.8	2.6	2
Rodentia, Chinchillidae	*Chinchilla laniger*	No	20.2	1.7 (4.4)	11.2 (67.2)	7.3 (28.2)	6.4	1.5	3
Rodentia, Caviidae	*Kerodon rupestris*	No	19.2	5.3 (16.6)	6.8 (49.6)	6.6 (30.9)	5.2	1.3	2
	Cavia porcellus	No	17.5	4.8 (17.6)	5.7 (48.1)	6.3 (34.2)	4.5	1.1	6,7
Rodentia, Muridae	*Acomys cahirinus*	No	42.0	2.3 (3.2)	23.0 (75.7)	12.3 (26.0)	11.6	2.8	2
	Mus musculus	No	40.8	2.6 (3.1)	27.0 (74.6)	12.5 (22.2)	13.8	3.3	3
	Rattus norvegicus	No	22.1	3.8 (10.5)	8.8 (56.1)	8.1 (33.2)	6.0	1.4	3
Rodentia, Cricetidae	*Microtus montebelli*	No	23.3	1.6 (3.7)	11.8 (62.5)	9.9 (33.7)	7.2	1.7	8
Chiroptera, Vespertilionidae	*Myotis lucifugus*	Yes	27.1	4.0 (7.6)	15.8 (70.3)	8.5 (24.4)	8.6	2.0	4
	Myotis velifer	Yes	32.4	4.4 (6.8)	19.9 (71.4)	10.7 (24.6)	10.6	2.5	4

Note. Energy content of milk (kcal/g) was estimated from energy equivalents of protein, sugars, and fat by the equation $E = 9.11F + 5.86P + 3.95S$, where the units for fat, protein, and sugars are grams per gram of whole milk as in Derrickson et al. (1996).

*References: 1. current study; 2. Derrickson et al. (1996) ; 3. Oftedal and Iverson (1995); 4. Kunz et al. (1995); 5. Veloso et al. (2003); 6. Oftedal (1981); 7. Nelson et al. (1951); 8. Sugawara et al. (1990).

Table 8. Comparison of milk mineral composition (mg/ml) at mid-lactation for several rodents.

Taxon	Species	Ca	K	Mg	Na	P	Ref*
Rodentia, Sciuridae	*Urocitellus columbianus*	4.7	1.4	0.23	0.86	3.9	1
Rodentia, Muridae	*Mus musculus*	3.6	1.1	0.24	0.84	2.2	2
	Rattus norvegicus	1.0	---	0.16	---	---	3
Rodentia, Cricetidae	*Microtus montebelli*	2.9	---	---	---	---	4
Rodentia, Caviidae	*Cavia porcellus*	2.3	0.89	0.27	0.47	1.2	5

Note. Concentrations are given as mg/ml unless otherwise specified.*References: 1. current study; data are mg/g (approximately equivalent to mg/ml); 2. (Yajima et al. 2006); 3. (Keen et al. 1981); 4. (Sugawara et al. 1990); 5. (Anderson and Sheffield 1988).

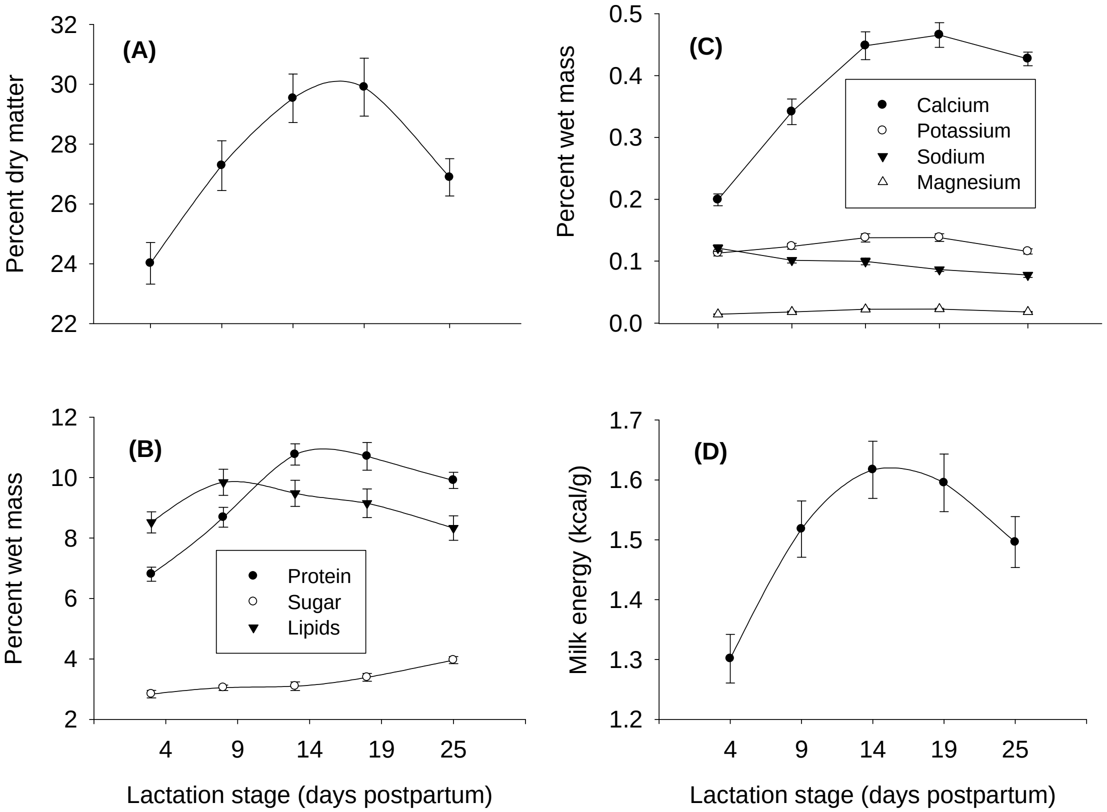

Figure 3. Temporal changes in the concentration of milk components. A) change in dry matter composition, B) change in proximate components, C) change in mineral composition, and D) change in gross energy content of milk. Error bars represent standard errors.

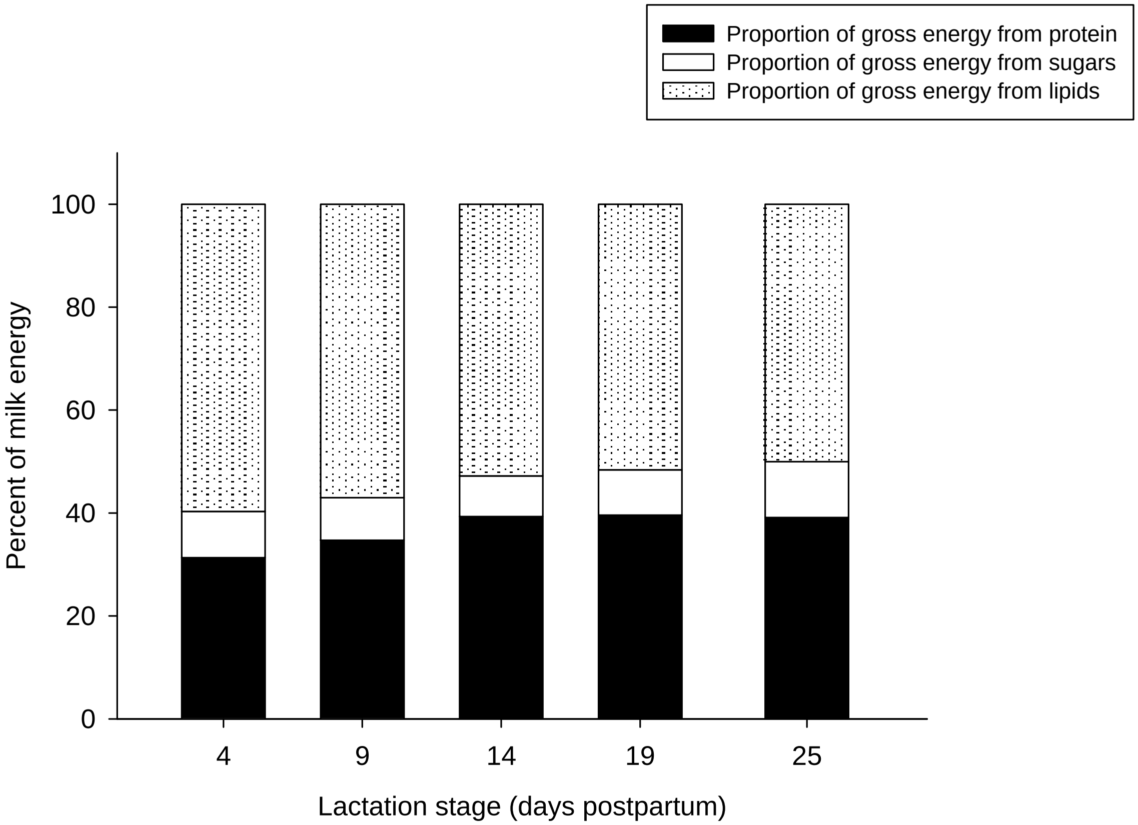

Figure 4. Contribution of lipids, protein and carbohydrates to gross energy content of milk throughout lactation. Gross energy was calculated by the equation $E = 9.11F + 5.86P + 3.95S$, where the units for fat, protein, and sugars are grams per gram of whole milk (Derrickson et al. 1996).

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CHAPTER THREE

MILK MATTERS: OFFSPRING SURVIVAL IN COLUMBIAN GROUND SQUIRRELS IS AFFECTED BY NUTRIENT COMPOSITION OF MILK

Abstract. Maternal effects are a means by which information about the environmental conditions experienced in the maternal generation can be transmitted to subsequent generations. Although maternal effects have been described and quantified in many mammalian species, the underlying mechanisms involved are often under-studied. The close association between mother and neonate during the extended period of lactation in mammals provides a unique opportunity for mothers to influence their offspring through nutrient provisioning via milk. The purpose of this study was to examine relationships between a female's size and reproductive timing and the nutrient composition of milk she produces, and the effects of inter-individual variation in milk composition on differential success of juveniles. Variation among females in lipid and energy content of milk was related to timing of reproduction. Females with intermediate birth dates produced milk that was higher in total lipid and energy content than females with early or late birth dates. In turn, the fat and energy content of milk had a positive effect on the probability of juvenile survival over-winter. The percent difference in milk energy content of females with young surviving hibernation versus those without was 14% and females with surviving young produced milk that had 3% more milk fat than females without surviving

young. Our results indicate that the interplay between timing of reproduction and lactation performance has important consequences for maternal and offspring fitness.

INTRODUCTION

There is overwhelming evidence that a mother’s phenotypic characteristics can impact the phenotype of her offspring, independent of direct genetic effects. These so called maternal effects are ubiquitous in nature occurring across many taxa from maternal influences on seed mass and dispersal patterns in plants (Donohue and Schmitt 1998) to the impact of maternal rank on offspring growth and age at reproductive maturity in mammals (reviewed in Holekamp and Dionak 2009). These effects can occur through two broad mechanisms- nutritionally via food provisioning and non-nutritionally, such as maternal care behaviors or the transfer of antibodies or hormones from mother to young. Such effects can alter behavioral, physiological, and/or morphological traits of offspring. Through these mechanisms maternal effects contribute to trans-generational phenotypic plasticity whereby offspring phenotype can be modified in response to environmental conditions occurring in the maternal generation (Fox and Mousseau 1998). Maternal effects also have a genetic component, providing an additional source of genetic variation upon which natural selection can act (Kirkpatrick and Lande 1989, Lande and Kirkpatrick 1990, Wolf et al. 1998). Thus, maternal effects can be prevalent forces in adaptation to new environmental conditions and in the evolutionary dynamics of populations (Bernardo 1996).

Maternal effects in mammals are expected to be especially prevalent because of the extended period of offspring dependence occurring during lactation (Maestripieri and Mateo 2009). Milk provides young with the building blocks required to support tissue growth prior to independence from the mother (Robbins et al. 1981). Undernutrition during early postnatal life can retard postnatal development and can have negative effects on physiology into adulthood. For example, low intake of specific nutrients affects neural development (Morgan and Naismith 1982), can result in skeletal maladies (Flynn 2003, Prentice 2004), and can alter glucose homeostasis into adulthood (de Souza and Moura 2000). Thus, the quality of offspring produced is at least partly dependent on a female's ability to deliver the appropriate nutrients in milk (Robbins et al. 1981).

In numerous species of mammals, the reproductive performance of individual females, measured as offspring growth, mass, litter size, or survival has been shown to vary with maternal characteristics, such as age, parity, body condition, body size, and reproductive timing (Clutton-Brock et al. 1982, Hoogland 1995, Skibiel et al. 2009). Larger females in better body condition tend to invest more resources into reproduction (Price 1998, Broussard et al. 2005), either producing more young or heavier individual young. In addition, females that reproduce earlier within the reproductive period tend to have greater success (Clutton-Brock et al. 1982, Dobson and Michener 1995, Rieger 1996, Huber et al. 1999, Neuhaus 2000). Breeding earlier may be advantageous due to reduced competition among females for high quality food sources (Clutton-Brock et al. 1982) or it may afford females more time to gain somatic stores in preparation for subsequent breeding attempts (Cohen 1976).

Despite the plethora of studies addressing patterns of reproductive investment in both ecological and evolutionary contexts, relatively little is known about the underlying link between variation in female size, condition, and reproductive timing and reproductive performance. Nevertheless, there is some evidence that a mothers somatic tissue stores can impact the amount of nutrients that she transfers to her young in milk. Milk fat content has been shown to vary with body fat stores in humans (*Homo sapiens*;

Nommsen et al. 1991, Dewey 1997), subantarctic fur seals (*Arctocephalus tropicalis*; Georges et al. 2001) and Rhesus macaques (*Macaca mulatta*; Hinde 2007). Female size and body fat also have effects on milk fat and energy content in common marmosets (*Callithrix jacchus*; Tardif et al. 2001) and mares (Doreau et al. 1992) and on total milk fat and energy produced over the course of lactation in the gray seal (*Halichoerus grypus*; Mellish et al. 1999). In turn, composition of milk, especially fat and energy content, has been demonstrated to have impacts on postnatal growth rate and body mass in seals, ungulates, and primates (Iverson et al. 1993, Mellish et al. 1999, Landete-Castillejos et al. 2001, Tardif et al. 2001).

In this study, we assess factors that contribute to variation in maternal investment in milk production and impacts on offspring size, growth, and survival in the Columbian ground squirrel *Urocitellus columbianus*. This is an ideal species for studying factors that contribute to and the consequences of inter-individual variation in milk composition for several reasons. First, it is possible to collect adequate volumes of milk to quantify its constituents. Second, because all individuals in the population can be observed throughout the active season and upon emergence the following spring, survival following hibernation can easily be determined. Third, the physiological abilities of individual squirrels are tested annually during hibernation. Unlike most other hibernators whose preparation for hibernation occurs well after reproductive investment ceases, young Columbian ground squirrels enter hibernation shortly after weaning (Dobson and Murie 1987, Young 1990, Dobson et al. 1992). After birth, pups consume only the mother's milk during the 4 week lactation period. Young are weaned just a few weeks before hibernation commences and then they must fast for 8-9 months during hibernation

(Dobson and Murie 1987, Dobson et al. 1992). Under these conditions, the consumption of milk with nutrient concentrations within a specific range is expected to be critical for offspring growth and survival. Therefore, interactions between maternal and offspring phenotype through lactation performance are expected to be especially pronounced in this species.

Previous studies of Columbian ground squirrels have shown that body size, body condition, and reproductive timing impact reproductive performance (King et al. 1991, Dobson et al. 1999, Skibiel et al. 2009). Specifically, larger females in better condition produce either heavier young that grow faster during the lactation period or more young (King et al. 1991, Dobson et al. 1999, Skibiel et al. 2009), and females that reproduce earlier have more young that survive hibernation (Dobson et al. 1999). If these relationships are mediated through nutritive properties of the milk produced, larger females in better body condition and/or those that reproduce earlier in the season are expected to produce milk with higher concentrations of fat, protein, and energy. Supplementing Columbian ground squirrel populations with a high protein feed results in heavier individuals and higher survival rates of juveniles through their first hibernation (Dobson and Kjelgaard 1985). Thus, we expected that females provisioning young with high protein milk would produce young with faster growth rates and higher weaning masses and a greater probability of survival over-winter. Furthermore, juveniles and adults rely on internal fat stores as the primary energy source during hibernation (Tahti 1978, Serkova et al. 2007). Therefore, young consuming higher fat milk are expected to be heavier and have a greater chance of survival.

METHODS

Field and *laboratory methods*

We studied a population of Columbian ground squirrels inhabiting Meadow Dot (N 50°38'59.6" W 114°39'40.9", elevation 1565 m) in the Sheep River Provincial Park (Alberta, Canada) in 2009. All squirrels were captured upon emergence from over-winter hibernacula with Tomahawk Live Traps (Tomahawk #201 collapsible chipmunk trap, 48 x 15 x 15 cm, Tomahawk, Wisconsin, USA) baited with a small amount of peanut butter. We tagged animals with fingerling eartags containing a unique number for permanent identification, we weighed them with a spring-loaded scale (Pesola Ag, Baar, Switzerland), and we measured zygomatic arch breadth using dial calipers (Swiss Precision Instrument, Garden Grove, California, USA). Animals were also painted with a unique marking on their backs using black hair dye (Lady Clairol Hydrience #51 Black Pearl; Proctor and Gamble, Stamford, CT, U.S.A.) in order to observe individuals at a distance. Mating dates of all females were determined by examining reproductive morphology and through observations of mating behavior. Twenty-four days were added to the known mating date to estimate parturition date (Shaw 1925, Murie and Harris 1982).

Two to three days before the estimated parturition date, we captured the females and transported them to the laboratory at the field station approximately 0.75 km from the field site. Females were housed individually in polycarbonate rat cages (267 x 483 x 20 mm^3; Allentown Caging Equipment Company, Allentown, New Jersey, USA) containing pine chips and newspaper and covered in vented black plastic to reduce stress. Females were maintained on horse feed (EQuisine sweet show horse ration, Unifeed, Okotoks, Alberta, Canada) *ad libitum* and lettuce and apple fed twice daily as the sole water source. We checked cages 3-4 times per day for visual signs of neonates. Approximately 6 hours following first observation of pups in the cage, initial post-partum measurements and records were taken. This included female mass and offspring mass and sex. For identification purposes, we removed a small portion of tissue from the outer toe bud of the left or right hind foot of all neonates. Mother and pups were released back on the meadow to their natal burrows 1-2 days postpartum.

We captured all females and juveniles when the litter emerged from the natal burrow around the time of weaning (approximately 27 days postpartum; Murie 1992). Juveniles were weighed, tagged, and marked using the same methods as for the adults earlier in the spring. Survival of juveniles over-winter was determined based on emergence the following spring. Columbian ground squirrels exhibit male-biased dispersal, which does not occur until after the first hibernation (Boag and Murie 1981, Murie and Harris 1984, Dobson and Murie 1987). Thus, juveniles that did not emerge the following spring were assumed to have died.

Milk collection and analysis

We collected milk approximately every 5 days postpartum (4, 9, 14, 19, and 25 days postpartum). Overall 36 females were milked; however, 8 females lost their litters at some point during lactation. For 28 females we have all 5 milk samples, but sample volumes were not always large enough to assay for all milk constituents. Females were captured on the meadow and held for 3 hours prior to milk collection to allow milk accumulation within the gland. Females were anesthetized with isoflurane, the nipples were cleaned, and oxytocin administered to stimulate milk letdown. Milk was expressed by light manual palpation, transferred to screw top vials, and stored at -80°C until analyses could be completed.

We assayed milk for proximate composition (i.e. fat, protein, and sugars) following methods for micro volumes of milk (Hood et al. 2009). Dry matter was determined by drying to constant mass, total lipid by micro-modification of the Roese-Gottlieb method, sugar content with the phenol-sulfuric acid method and crude protein by CHN elemental analysis. Gross energy content of milk was estimated from energy equivalents of proximate components of cow's milk as in Derrickson et al. (1996). All data on proximate milk composition are presented as a percent of wet mass whereas gross energy content is expressed as kcal/g.

Variables

Milk composition was examined relative to female characteristics including structural size, body condition, and timing of reproduction. Zygomatic arch breadth, measured at spring emergence from hibernation, was used as an estimate of structural size. The julian date (1 January = day 1) of litter birth (hereafter referred to as parturition date) was used as a measure of reproductive timing. Body condition was estimated by extracting the residuals from the regression of spring emergence mass on zygomatic arch breadth (Dobson et al. 1999, Georges et al. 2001, Schulte-Hostedde et al. 2005, Skibiel et al. 2009). Measures of reproductive performance included offspring weaning mass, growth rate during lactation and survival over-winter. Growth rate was calculated by subtracting mass at birth from mass at weaning and dividing by lactation duration to yield mass gain in grams per day. When litter size was greater than 1, weaning mass and growth rates were averaged within litter.

Statistics

SAS statistical software (SAS 2002) was used to run all statistical analyses. Shapiro-Wilks tests were employed to determine if data were normally distributed. To examine effects of female size, body condition, and parturition date on milk composition (e.g. protein, fat and sugar concentration and gross energy content), we used repeated measures MANOVAs (PROC GLM) with the categorical variable day (5 levels corresponding to day of sample collection: 4, 9, 14, 19, and 25) included as the repeated measure. We first plotted each maternal characteristic against the milk constituents to assess linearity of the relationships. Parturition date appeared to exhibit a curvilinear relationship with the milk constituents and thus parturition date was also included in the models as a squared term.

Repeated measures ANCOVAs (PROC GLM) were used to examine impacts of milk composition on offspring weaning mass and growth rate including day (of sample collection) as a repeated measure. Litter size at weaning is significantly negatively correlated to offspring weaning mass and growth rate (Skibiel et al. 2009) and was therefore included as a covariate. Repeated measures logistic regression (PROC GENMOD) was used to examine effects of milk composition on the probability of that female having at least 1 pup survive over-winter. Female ID was included as the repeated measure to account for multiple milk samples collected for each female. Only 2 females had more than 1 pup survive over winter and both of these females had 2 pups survive, thus we did not account for multiple pups within a litter except to include litter size at weaning as an additional predictor variable.

Although we performed each of these tests on all 4 milk constituents separately we did not use a Bonferroni correction to correct the α-level for multiple comparisons. While decreasing the chance of a type I error, Bonferroni correction tends to be too conservative, increasing the probability of a type II error (Perneger 1998, Garamszegi 2006). This is problematic when biologically important findings are disregarded because they are statistically non-significant with a Bonferroni correction. Thus, α-level was 0.05 for all statistical analyses.

RESULTS

Female mass at spring emergence exhibited a positive and significant relationship with zygomatic arch breadth ($R^2 = 0.69$, $n = 50$, $P < 0.0001$). Residuals extracted from these regressions were normally distributed ($W = 0.98$, $n = 50$, $P = 0.53$) and thus were used as an estimate of body condition. Averaged across all sample days, lipid concentration of milk was 9.0 ± 1.4%, sugar was 3.2 ± 0.5%, protein was 9.2 ± 1.3% and milk energy density was 1.5 ± 0.2 kcal/g.

There was a significant curvilinear relationship between parturition date and milk lipid concentration (parturition date: $F_{1,21} = 11.17$, $P = 0.003$; parturition date^2: $F_{1,21} = 11.17$, $P = 0.003$) and gross energy (parturition date: $F_{1,21} = 10.91$, $P = 0.004$; parturition date^2: $F_{1,21} = 10.98$, $P = 0.004$). This was due to an effect of parturition date on milk lipid concentration at peak lactation (day 19 postpartum) and day 25 postpartum (day 19, parturition date: $F_{1,21} = 10.14$, $P = 0.005$; parturition date^2: $F_{1,21} = 10.33$, $P = 0.004$; Fig. 5; day 25, parturition date: $F_{1,21} = 5.05$, $P = 0.03$; parturition date^2: $F_{1,21} = 5.12$, $P = 0.03$)

and on gross energy content at day 19 and 25 postpartum (day 19, parturition date: $F_{1,21}$ = 6.24, P = 0.02; parturition date^2: $F_{1,21}$ = 6.24, P = 0.02; day 25, parturition date: $F_{1,21}$ = 8.08, P = 0.01; parturition date^2: $F_{1,21}$ = 8.18, P = 0.01). There was no effect of maternal condition or size on any milk constituents (all $P > 0.05$). Litter size had a significant negative effect on weaning mass and growth rate for all models ($P < 0.05$). After statistically controlling for effects of litter size on offspring characteristics, only sugar concentration at 9 days postpartum had a significant positive effect on weaning mass and growth rate (weaning mass: $F_{1,19}$ = 8.94, P = 0.008; growth rate: $F_{1,19}$ = 6.71, P = 0.02). Females who produced milk higher in sugar content at 9 days postpartum (n = 27, Z = -2.04, P = 0.04), milk higher in fat concentration at 19 days postpartum (n = 26, Z = -2.64, P = 0.008; Fig. 6), and milk with higher energy content at 25 days postpartum (n = 26, Z = -2.07, P = 0.04) had a greater probability that at least one of her pups would survive overwinter. There was also a trend for an effect of milk fat concentration at day 25 on probability of survival (n = 26, Z = -1.84, P = 0.06). Females who had at least one juvenile survive produced milk with an average of 3.2 ± 0.38% sugar at 9 days postpartum, 10.3 ± 2.3% fat at 19 days postpartum and 9.1 ± 2.2% fat and 1.6 ± 0.2 kcal/g of energy at 25 days postpartum whereas females who did not have any juveniles survive produced milk with an average of 2.9 ± 0.58% sugar at 9 days postpartum, 7.7 ± 1.9% fat at 19 days postpartum and 7.3 ± 1.8% fat and energy content of 1.4 ± 0.2 kcal/g at 25 days postpartum. Litter size at weaning did not have a significant effect on probability of survival (all $P > 0.1$).

DISCUSSION

Maternal characteristics and milk composition

None of the milk constituents examined varied with maternal condition or size, suggesting that the relationship between body condition and size of Columbian ground squirrel females and offspring weaning mass described in Skibiel et al. (2009) is unlikely to be attributable to milk composition. Larger females in better condition are expected to allocate more resources to reproduction (Price 1998, Broussard et al. 2005) because they may be better able to compete for high quality resources (Ralls 1976) or because they have greater internal stores of energy (Iverson et al. 1993). Females of larger size or females in better condition may produce larger young not only by altering nutrient composition of milk, but by producing larger volumes of milk or by increasing the amount of time spent suckling young. Rejection rates, amount of time spent in contact with the infant, and suckling frequency have all been shown to vary with maternal condition in primates (Gomendio 1989, Fairbanks and McGuire 1995). In Holstein cows, females with a greater body condition score produced greater milk yields (Domecq et al. 1997) and heavier female grey seals with larger endogenous protein reserves had greater daily milk outputs and longer lactation lengths (Mellish et al. 1999). Milk yield measurements are based on isotope dilution in the young, isotope transfer between the mother and offspring, or change in the mass of young following a suckling event (Hood et al 2009). In all cases access to the young is essential for completing these measurements. Because the dependent Columbian ground squirrel pups reside in underground burrows during the lactation period, it was not possible to measure milk intake for this species.

Timing of reproduction has significant impacts on reproductive performance of fish, mammals, and birds (Festa-Bianchet 1988, Verhulst and Tinbergen 1991, Dobson and Michener 1995, Einum and Fleming 2000) and may be especially critical for hibernating animals that need to gain fat mass prior to hibernation. By mating earlier, females and juveniles have more time to acquire fat reserves before hibernation commences (Murie and Boag 1984, Millesi et al. 1999). We found that variation among female Columbian ground squirrels in the timing of parturition contributed to variation in milk lipid concentration and gross energy from peak to late lactation. Although births spanned a period of only 3 weeks, females that gave birth near the beginning and the end of the birthing period had lower milk lipid and energy content than females that gave birth around the median parturition date (Fig. 5). This phenomenon could arise if females giving birth around the median birth date entered peak lactation coinciding with the time of greatest nutritive quality of food resources. This is not likely for our population, however, as females giving birth around the median birth date would have been in peak lactation in mid-June and crude protein and digestibility of grasses and forbs on meadows in the Sheep River Provincial Park decline from early June to mid-August (Bennett 1999). Alternatively, females reproducing earlier may restrain investment in energy dense milk as young may have adequate time to compensate post-weaning whereas females breeding later in the season may face constraints associated with the necessity to gain mass in preparation for hibernation. In Iberian red deer (*Cervus elaphus hispanicus*), artificial advancing of calving date resulted in hinds producing milk with lower fat concentration and those hinds weaned their calves earlier (Gomez et al. 2002). Calving earlier allowed more time for growth between weaning and the onset of winter and as a result earlier born calves had faster growth rates post weaning (Gomez et al. 2002). Further studies are required to determine if a similar scenario is occurring within Columbian ground squirrel populations.

Impacts of milk composition on offspring

We found that milk sugar concentration at 9 days postpartum had a significant positive effect on offspring weaning mass, growth rate, and probability of juvenile survival. Milk lactose content is also associated with offspring growth rates in Iberian red deer (Landete-Castillejos et al. 2001). Sugars, along with minerals, are involved in osmolarity of milk, where greater concentration of sugars within mammary alveoli osmotically retains water within the mammary gland (Shennan and Peaker 2000). Thus, greater concentrations of lactose will also result in secretion of greater milk volumes.

Surprising is the lack of association between milk lipid, protein, and energy content on offspring weaning mass and mass gain during the lactation period in Columbian ground squirrels. Offspring mass and growth rates are correlated to protein, fat, and energy intake in several mammals including gray seals (Iverson et al. 1993, Mellish et al. 1999), Iberian red deer (Landete-Castillejos et al. 2001, Gomez et al. 2002), and common marmosets (Tardif et al. 2001). Mass and growth, however, are dependent not only on milk composition, but also on lactation length (Mellish et al. 1999) and milk yield (Landete-Castillejos et al. 2003, Hinde et al. 2009), which together determine the total amount of nutrients transferred during the lactation period, and the neonate's metabolic efficiency in converting nutrients and energy into mass (Mellish et al. 1999).

Females that produced milk averaging 3% more lipids and 0.2 kcal/g more energy were more likely to have at least one offspring survive over-winter than those with lower milk fat and energy content (Fig. 6). In Columbian ground squirrels, surviving the first hibernation is critical as most deaths occur during this time period (Bennett 1999) and greater juvenile mass and growth rates are associated with a higher probability of survival through the first hibernation (Murie and Boag 1984, Skibiel et al. 2009). This relationship, however, does not appear to be related to the lipid, protein, or energy content of milk as none of these milk constituents were related to mass or growth. Polyunsaturated fatty acids are an essential component of the diet particularly for hibernating species (Frank 1992, Florant et al. 1993). It is possible that small differences in milk fat were attributed to vital differences in milk fatty acid composition, and possibly other lipid-soluble compounds, that may have contributed to differences in offspring survival independent of differences in offspring mass and growth rate.

Milk composition could also influence survival by affecting juvenile physiology or behavior. The early environment experienced by young, including consumption of nutrients, antibodies, and hormones through milk, can have profound impacts on neonatal physiology from immune function to glucose metabolism (Holness and Sugden 1996, Hasselquist and Nilson 2009). In rhesus macaques, infants consuming higher energy milk had higher activity levels, potentially occurring through postnatal impacts of milk energy transfer on neural development (Hinde and Capitanio 2010). For juvenile Columbian ground squirrels, maternal effects occurring during the lactation period have significant effects on over-winter survival (Skibiel et al. 2009), but mass gain during the time of independent foraging post-weaning is also important for survival (Bennett 1999). Higher activity levels of juvenile Columbian ground squirrels may be advantageous in promoting greater foraging rates prior to hibernation. Investigating effects of milk composition on juvenile physiology and behavior would be worthy endeavors for future research.

CONCLUSIONS

Timing of reproduction appears to be an important factor contributing to variation in milk composition as females that gave birth at either end of the birthing period produced milk lower in fat and energy. Milk higher in these components promotes over-winter survival of juveniles. Reproducing earlier, however has been shown to have a positive effect on probability of juvenile over-winter survival in this species (Dobson et al. 1999, Neuhaus 2000), suggesting that despite the lower fat and energy in milk produced, it is still advantageous to reproduce early. This likely occurs because young have more time post-weaning to forage independently and gain fat mass prior to hibernating. The results of this study underscore the need to examine the mechanisms through which maternal effects occur and their influences on fitness.

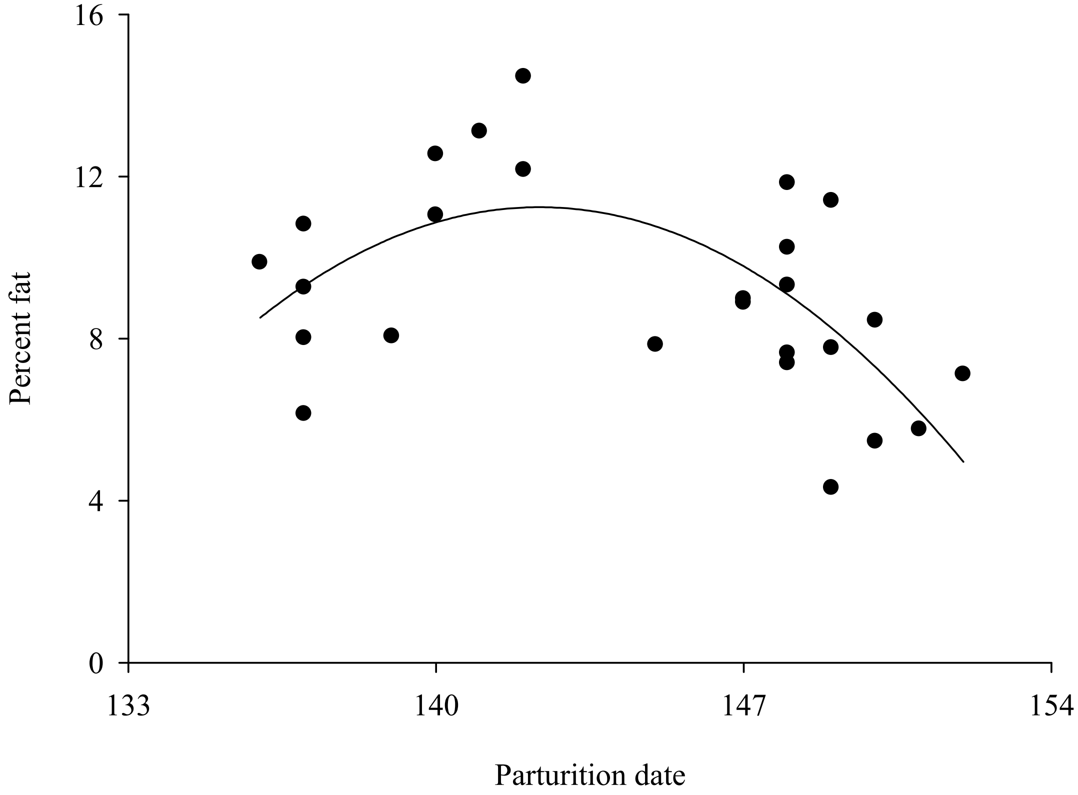

Figure 5. Relationship between fat concentration of milk at 19 days postpartum and parturition date. The relationship between milk energy and parturition date were the same as for fat concentration and is therefore not presented here.

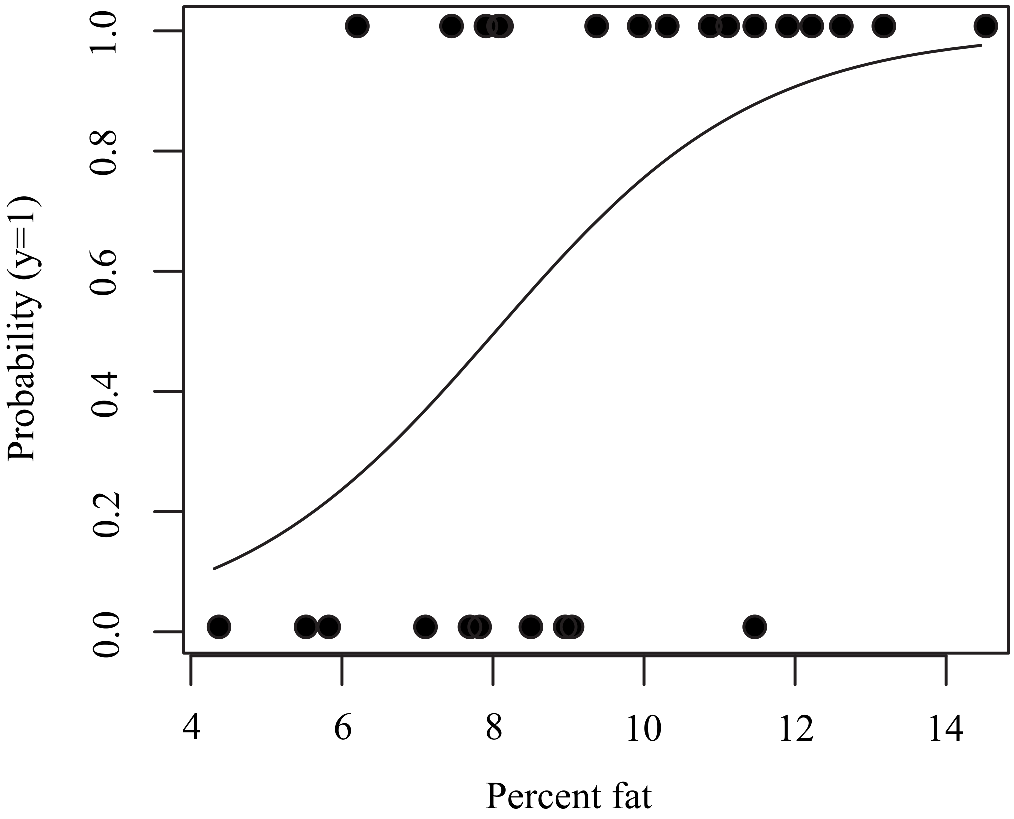

Figure 6. Relationship between milk fat concentration at 19 days postpartum and the probability that a female had at least one pup survive over-winter. A probability of 1.0 indicates that a female had at least one pup survive over-winter whereas a probability of 0.0 indicates that the female did not have any pups survive over-winter. Females with greater milk fat concentration had a greater probability of having surviving offspring (*n* = 26, *Z* = -2.64, *P* = 0.008).

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CHAPTER FOUR

COLUMBIAN GROUND SQUIRRELS INCUR AN ENERGETIC COST TO LACTATION WITHOUT AN EFFECT ON MATERNAL SURVIVAL OR FUTURE REPRODUCTION

Abstract. Life history evolution is contingent upon proximate and ultimate costs of reproductive effort. Allocating a greater amount of limited resources, such as energy, to current reproduction can reduce the amount of energy available for somatic maintenance and can ultimately impair future breeding success or maternal survival (i.e. cost of reproduction hypothesis). Although there is some support for the cost of reproduction in birds, few empirical studies of mammals have demonstrated a tradeoff between current and future reproduction. Furthermore, most studies assume a proximate energetic cost to reproduction that may or may not be present. We experimentally manipulated litter size in a wild population of Columbian ground squirrels for 2 years to examine the energetic and fitness (i.e. survival and breeding) costs of reproduction. Although females raising augmented litters had field metabolic rates that were almost 1.5 times greater than females raising control or reduced litters, there were no negative impacts on the probability of maternal survival or future reproduction. However, pups from augmented litters grew more slowly during the lactation period, were smaller at weaning and had a lower probability of survival over-winter. Thus, although females are capable of raising more young than they give birth to, the reduced offspring survival associated with raising larger litters precludes any benefit of weaning a larger litter.

INTRODUCTION

Fundamental to our understanding of the evolution of life histories is an evaluation of the proximate physiological and ultimate costs associated with maternal investment in reproduction. Maternal investment is an important component of mammalian reproductive strategies and includes any characteristic or behavior that is likely to enhance offspring survival but entails a cost to the mother (Trivers 1972). Time, energy, and nutrients invested in the current litter may reduce the resources that females can allocate to self-maintenance; thus, investment can reduce the availability of maternal resources necessary to support future fecundity and even survival (cost of reproduction tradeoff; Williams 1966a, Williams 1966b, Bell 1980). An implicit assumption of this life history tradeoff is that resources are finite and thus individuals must allocate limited resources among multiple competing processes. Associated with relative demand, the costs of reproduction are expected to be most pronounced in females supporting more young (Reznick, 1985, Godfray et al., 1991, Daan et al., 1996, Sikes and Ylönen, 1998).

Although there is some support for the cost of reproduction tradeoff in birds (Dijkstra et al. 1990 and references therein), relatively few studies have shown a decrease in maternal survival or fecundity with increasing litter size in mammals (Festa-Bianchet et al. 1998a, Koivula et al. 2003). Many studies on free-ranging mammals have been correlative where a measure of current reproduction, such as litter size or mass, is correlated to a female's survival or to the mass or size of the next litter produced (e.g. Murie and Dobson 1987, Risch et al. 1995). Experimental manipulations of litter size are preferable because correlations could be due to a common third variable not tested (Partridge and Harvey 1985) and because natural litter size does not necessarily reflect reproductive capability (Reznick 1985). Although brood size manipulations are common in birds, they are infrequently conducted in free-ranging mammals, likely due to the inability to access young or because females are capable of discriminating between their own and fostered young. However, some small mammals readily accept cross-fostered young, such as bank voles (*Clethrionomys glareolus*; Koivula et al. 2003, Mappes and Koskela 2004), red squirrels (*Tamiasciurus hudsonicus*; Humphries and Boutin 2000, McAdam et al. 2002), and Columbian ground squirrels (*Urocitellus columbianus*; Hare and Murie 1992, Murie et al. 1998, Neuhaus 2000).

More recently, physiological mechanisms underpinning the cost of reproduction tradeoff have gained greater attention; yet we still know relatively little about the physiological costs of reproduction and their fitness impacts. One potential physiological cost likely to be involved in the tradeoff between current and future reproduction is maternal energy expenditure during reproduction (Speakman 2008). Reproduction, particularly lactation, is the most energetically expensive period of a female's life, due in large part to the high metabolic demands of milk production (Oftedal 1984, Racey and Speakman 1987, Gittleman and Thompson 1988). Although energy intake or energy expenditure have been quantified for many female mammals raising litters of varying sizes (Randolph et al. 1977, Glazier 1985, Atramentowicz 1992, Hammond and Diamond 1992, Humphries and Boutin 2000, Johnson et al. 2001), few investigate the impacts of energy investment in the current litter on a female's future fecundity or survival. Furthermore, most studies on the energetics of reproduction are conducted on captive or domestic animals and information on reproductive costs gleaned from these studies cannot be extrapolated to free-ranging animals. This is because reproductive costs will likely be substantially different in the field due to increased activity, variation in resource availability and quality, the presence of predators, atmospheric conditions, and interactions with conspecifics (Nagy 1987, Stearns 1992, Berteaux 1998).

The objective of this study is to investigate the proximate energetic and ultimate costs of investment in lactation by artificially manipulating litter size by two pups in a free-ranging population of Columbian ground squirrels, *Urocitellus columbianus* (CGS). We tested for reproductive costs by examining effects of litter size manipulation on weaning success and offspring survival to yearling age, on female energy expenditure, mass change, and on female survival and breeding the following year. If energetic investment in the current litter entails a reproductive cost, we expect females raising larger litters to have higher energy expenditures, greater mass loss during lactation and a lower probability of survival or breeding the following year.

The Columbian ground squirrel is a great species for addressing fitness related costs of energy expenditure during lactation for several reasons. First, Columbian ground squirrels are easy to trap and handle repeatedly, making them ideal candidates for use of the doubly labeled water method to determine energy expenditure. To the best of our knowledge, this is the first study reporting field metabolic rates in this species. Second, energetic costs of reproduction are expected to be significant when there is limited time for regaining depleted energy sources before they are needed again (Humphries and Boutin 2000). Columbian ground squirrels have a short active season before entry into hibernation and pups are weaned just a few weeks before hibernation begins (Young 1990, Dobson 1992). This limits the amount of time females have post-weaning to gain somatic fat deposits, which the squirrels depend on for energy during hibernation (Murie and Boag 1984, Young 1990, Dobson et al. 1992). Furthermore, females reproduce within just a few days of emerging from hibernation the next spring (Murie and Harris 1982) limiting the amount of time females have to gain mass prior to the beginning of gestation. Thus, if there are energetic costs to reproduction, they should be detected in this species. Third, unlike many other species of rodents, we are able to obtain survival rates for mothers and juveniles because Columbian ground squirrels exhibit a delayed (i.e. occurs after juveniles' first hibernation) and male-biased dispersal. Finally, previous studies failed to find evidence of reproductive costs when litter size was augmented by one pup (Hare and Murie 1992, Neuhaus 2000). Female metabolic rate was not measured in these studies so there are no data to confirm that the additional pup was energetically costly to females. After initial start-up costs of offspring production are paid for, the additional energetic cost of lactation for an increase of one pup is relatively small, particularly for intermediate litter sizes (Sikes 1998) indicating that females may be easily able to compensate for raising just a single additional pup. Therefore, adjusting litter size by more than one pup might be more effective in elucidating reproductive costs.

METHODS

A population of Columbian ground squirrels inhabiting Meadow Dot (N 50°38'59.6" W 114°39'40.9", elevation 1565 m) in the Sheep River Provincial Park, Alberta, Canada was studied from 2009-2011. We trapped all squirrels on the meadow at spring emergence from hibernation using Tomahawk live traps (Tomahawk #201 collapsible chipmunk trap, 48 x 15 x 15 cm, Tomahawk, Wisconsin, USA) baited with peanut butter. Squirrels' ears were fitted with numbered fingerling tags for permanent identification and unique dye (Lady Clairol Hydrience #51 Black Pearl; Proctor and Gamble, Stamford, CT, USA) markings on their backs in order to observe individuals from a distance. Spring emergence mass for all animals was measured using a Pesola spring scale (Pesola Ag, Baar, Switzerland) and zygomatic arch breadth using dial calipers (Swiss Precision Instrument, Garden Grove, California, USA). Mating dates of all adult females were determined by visual observation of mating behaviors and examination of the degree of vaginal opening.

Two to 3 days prior to the estimated parturition date (24 day average gestation; Shaw 1925, Murie and Harris 1982), all females that mated were transported to the laboratory on site to give birth. Females were individually housed in polycarbonate microvent rat cages (267 x 483 x 20 mm^3; Allentown Caging Equipment Company, Allentown, New Jersey, USA) containing pine chips and strips of newspaper for nesting material. Cages were covered in vented black plastic bags to simulate the burrow environment and to reduce stress. Squirrels were fed lettuce and apples twice daily and horse feed (EQuisine sweet show horse ration, Unifeed, Okotoks, Alberta; oats, barley, wheat, and compressed vegetable material in a molasses mix) *ad libitum*. Cages were checked 3-4 times daily for the presence of pups and on the day of birth, the female and her litter were weighed, pups were sexed, and a small piece of tissue from the outer hind toe bud was removed for identification of individual neonates.

Females were paired based on parturition date (within 24 hours of each other) and randomly assigned to 1 of 3 treatment groups: litter augmented, litter reduced, or control. Pups within the litter were randomly chosen to either remain with its siblings or to be transferred to the paired litter. If the fostered pup was 3 g greater than or less than the average weight of its new littermates, the pup was reassigned. Litter augmented groups (A) received 2 additional pups (2009, $n = 9$; 2010, $n = 10$), litter reduced groups (R) had 2 pups removed (2009, $n = 9$; 2010, $n = 10$), and control groups (C-foster) had 1/3 to 1/2 of the litter cross-fostered (1-2 pups) so that litter size of those paired females remained the same as at birth (2009, $n = 14$; 2010, $n = 14$). Although there is no detrimental effect on offspring or females of the experimental procedure itself (Murie et al. 1998), cross-fostering in control groups was done as an extra precaution to ensure the procedure *per se* did not have an influence on reproductive performance. In some cases, only 1 female gave birth within a 24 hour period and thus could not be paired with another female for treatment. When this occurred that female was considered as a non-cross-fostered control (C-non-foster; 2009, $n = 5$; 2010, $n = 6$). Mothers and pups were then released back on the meadow to their nest burrows 1-2 days following parturition. Litter size was manipulated in some of the same females in 2009 and 2010 ($n = 30$); however, all females were randomly assigned to treatment groups without respect to treatment the previous year.

Daily energy expenditure (DEE) of females during peak lactation was estimated as field metabolic rate using the doubly-labeled water (DLW) method (Lifson and McClintock 1966, Speakman 1997). Field metabolic rate measured through DLW method during lactation incorporates energy associated with increased foraging rates and milk production but does not include the energy transferred to the young through the milk (Kenagy 1987, Kenagy et al. 1989, Kenagy et al. 1990). All females in the population that gave birth in the lab were captured on day 21 after parturition (day of parturition = day 0), weighed, and injected intraperitoneally with 0.5 ml of sterile DLW (10% atom percent excess APE-enriched ^{18}O and 99% APE-enriched ^{2}H mixed in a ratio of 20:1). Squirrels were held for 60 min to allow isotopes to equilibrate in the body following the rule of thumb of an equilibration time of 1 hour plus an additional hour for each 10 kg of body mass (Speakman 1997). After equilibration females were lightly anesthetized by placing them in a 4 L plastic container with a screw top lid containing cottonball pre-soaked in isoflurane. An initial blood sample was obtained by clipping the toenail to the quick. Females were released within 15 minutes of initial blood collection, recaptured 72 hours later, weighed, and anesthetized prior to taking a final blood sample. Isotope concentrations in the blood were determined following the methods of Ergon et al. (2004). CO_2 production was estimated using the single pool equation from Speakman (1997; Equation 7.17) and converted to daily energy expenditure (kJ/day) using a respiratory quotient of 0.85 (Speakman, 2007; Equation 8.3).

Females and pups were captured when pups first emerged from the nest burrows (average of 27 days post partum; Murie 1992), which approximates the time of weaning (Murie and Dobson 1987). Females and juveniles were weighed and juveniles were given uniquely numbered eartags and dye-markings. Juvenile growth rate during lactation was calculated by subtracting mass at birth from mass at weaning and dividing by the length of the lactation period. Female change in mass during lactation was calculated as the difference in mass between the time of litter emergence from nest burrows and immediately after giving birth. Growth rate and birth/weaning masses were averaged within litter (for all statistical tests except for differences between fostered and non-fostered young). Females and juveniles were assumed to have died if they were not seen emerging from burrows the following spring. This can be justified because males are the primary dispersers in this species and juveniles do not typically disperse until the spring following their first hibernation (Boag and Murie, 1981, Dobson and Murie, 1987, Murie and Harris, 1984).

Statistics

All statistical analyses were performed in SAS version 9.1.3 for Windows (SAS 2002). Data were examined for normality graphically and statistically by conducting Shapiro-Wilks tests. Differences between fostered and non-fostered young in the probability of survival were tested with logistic regression (PROC LOGISTIC) and in mass at weaning and growth rate with ANCOVA (PROC GLM) with year and litter size at weaning as covariates. There were no differences in offspring weaning mass, growth rate or survival to weaning between fostered and non-fostered young (weaning mass: $F_{1,42} = 0.20$, $P = 0.66$; growth rate: $F_{1,42} = 0.56$, $P = 0.46$; survival: $n = 75$, $\chi^2 = 0.14$, $P = 0.71$). Thus, we combined both cross-fostered and non-cross-fostered control groups into a single control group (C) for all subsequent analyses.

To determine if multiple records for females could be considered independent, we used Spearman rank correlations (PROC CORR spearman) to determine if reproductive performance in one year was related to reproduction the next year. For females whose litter sizes were manipulated in 2009 and 2010, we also tested whether treatment in 2009 had an effect on maternal and offspring traits in 2010 using ANCOVAs and if treatment in 2009 had an effect on whole or partial litter loss in 2010 using logistic regression with 2009 treatment and 2010 treatment as independent variables. Records for females in multiple years were considered independent for several reasons. First, empirical evidence suggests that in this species reproduction in one year is not related to reproduction in subsequent years (Murie and Dobson 1987, Risch et al. 1995, Dobson et al. 1999, Skibiel et al. 2009). Second, we found that measures of reproductive performance in 2009 were not related to the same measure in 2010 (litter size at birth, $n = 39$, $r = 0.29$, $P = 0.07$; average pup mass at weaning, $n = 39$, $r = 0.28$, $P = 0.08$; average pup growth rate, $n = 39$, $r = 0.29$, $P = 0.07$). Third, for females who were manipulated in both 2009 and 2010, treatment in 2009 had no effect on any offspring or maternal characteristics in 2010 (Table 9). In addition, treatment in 2009 had no effect on the probability of whole or partial litter loss in 2010 (whole litter loss: $n = 40$; 2009 treatment, $\chi^2 = 0.13$, *d.f.* = 2, $P = 0.93$; partial litter loss: $n = 40$; 2009 treatment, $\chi^2 = 0.42$, *d.f.* = 2, $P = 0.81$).

Differences among treatment groups in litter size at birth, after manipulation, and weaning, offspring mass at birth and weaning, offspring growth rates, and maternal traits the same year as the treatment were tested using two-way ANOVAs (PROC GLM) including year and treatment as independent variables. Breeding characteristics of females in the next year following treatment, including litter size and mass at parturition, mating date, spring emergence and parturition masses, were examined using ANOVAs including treatment and year of treatment as independent variables. When ANOVAs were significant, Tukey's tests for multiple comparisons were employed to determine which groups differed. Differences in the probability of litter loss, offspring survival, female survival, and breeding among treatment groups were tested with logistic regression including year and treatment as independent variables. Relationships between daily energy expenditure, mass-specific daily energy expenditure and subsequent maternal survival and breeding were determined by logistic regression with energy expenditure and year as independent variables. The relationship between treatment and whether a female gained, lost or maintained body mass was determined by fisher's exact test for a 2 x 2 contingency table (PROC FREQ). All tests with multiple independent variables also included an interaction term. Data presented as means include ± SE.

RESULTS

Litter size, offspring size, and offspring survival

Litter size at birth ranged from 1 to 5 ($\bar{x} = 2.8 \pm 0.08$); thus, enlarging litters by an additional 2 pups was a fairly substantial manipulation. Initial litter size at birth was different between treatment groups (treatment, $F_{2,73} = 9.70$, $P = 0.0002$; year, $F_{1,73} = 0.31$, $P = 0.58$; interaction n.s.) with females in the reduced treatment group having larger litter sizes at birth (Fig. 7). After the litter manipulation and at weaning, litter size still differed among treatment groups (after manipulation: treatment, $F_{2,73} = 128.66$, $P < 0.0001$; year, $F_{1,73} = 0.31$, $P = 0.58$; interaction n.s.; at weaning: treatment, $F_{2,73} = 21.77$, $P < 0.0001$; year, $F_{1,73} = 0.11$, $P = 0.74$; interaction n.s.). Females in the augmented group had the largest litter sizes at weaning (Fig. 7).

Pup mass at birth did not differ among treatment groups (treatment, $F_{2,73} = 1.67$, $P = 0.20$; year, $F_{1,73} = 0.04$, $P = 0.84$; interaction n.s) but pups in reduced and control groups were significantly heavier at weaning than pups raised in augmented litters treatment, $F_{2,55} = 12.03$, $P < 0.0001$; year, $F_{1,55} = 3.07$, $P = 0.09$; interaction n.s.; Fig. 8). Pup growth rate from birth to weaning also differed among treatment groups and pups grew faster in 2010 than in 2009 regardless of treatment (treatment, $F_{2,55} = 10.42$, $P = 0.0001$; year, $F_{1,55} = 4.65$, $P = 0.04$; interaction n.s.). Tukey tests revealed that rate of pup growth was significantly slower for pups raised in augmented litters than pups in either control or reduced litters (control, $n = 28$, $\bar{x} = 3.10 \pm 0.17$ g/day; reduced, $n = 15$, $\bar{x} = 3.54 \pm 0.24$ g/day, augmented, $n = 16$, $\bar{x} = 2.21 \pm 0.19$ g/day).

Overall 23% of females lost their whole litter during the lactation period whereas 18% of females lost at least one pup from birth to weaning. However, litter manipulation had no effect on the probability of whole or partial litter loss (whole litter loss, $n = 77$: treatment, $\chi^2 = 1.14$, *d.f.* = 2, $P = 0.57$; year, $\chi^2 = 0.52$, *d.f.* = 1, $P = 0.47$; interaction n.s.; partial litter loss, $n = 77$: treatment, $\chi^2 = 0.15$, *d.f.* = 2, $P = 0.93$; year, $\chi^2 = 0.22$, *d.f.* = 1, $P = 0.64$; interaction n.s.). The probability of individual offspring surviving to the next year was dependent on treatment (n = 151, treatment, $\chi^2 = 17.44$, *d.f.* = 2, $P = 0.0002$; year, $\chi^2 = 1.20$, *d.f.* = 1, $P = 0.27$; interaction n.s.). Pups raised in augmented litters (A) had a lower probability of survival than pups raised in either control (C) or reduced litters (R) (C vs. A: $\chi^2 = 11.29$, *d.f.* = 1, $P = 0.0008$; R vs. A: $\chi^2 = 14.31$, *d.f.* = 1, $P = 0.0002$).

Maternal characteristics

Female age, mass at parturition, and parturition date did not differ among treatment groups (Table 10). Females gave birth 5 days earlier on average in 2010 than in 2009 (treatment: $F_{2,73} = 1.24$, $P = 0.30$; year, $F_{1,735} = 18.86$, $P <0.0001$; interaction n.s.). Of the females that successfully weaned a litter, 53% lost mass from parturition to weaning ($n = 31$), 43% gained mass ($n = 25$), and 3% had no change in mass ($n = 2$). There was no significant relationship between treatment and whether a female gained, lost, or had no change in mass ($P = 0.17$). Female mass at weaning did not differ among treatment groups but females were heavier at weaning in 2009 than in 2010 regardless of treatment (2009, $\bar{x} = 514.6 \pm 9.3$ g; 2010: $\bar{x} = 485.2 \pm 7.9$ g; treatment, $F_{2,55} = 0.60$, $P = 0.55$; year, $F_{1,55} = 5.86$, $P = 0.02$; interaction n.s; Table 10). Females raising augmented litters had the highest daily energy expenditure and mass-specific daily energy expenditure (Table 10).

Maternal survival and fecundity

Treatment had no effect on the probability of female survival to the next spring ($n = 77$: treatment, $\chi^2 = 0.93$, $d.f. = 2$, $P = 0.63$; year, $\chi^2 = 0.78$, $d.f. = 1$, $P = 0.38$; interaction n.s.) or the probability of breeding the next year ($n = 58$: treatment, $\chi^2 = 0.003$, $d.f. = 2$, $P = 0.99$; year, $\chi^2 = 0.004$, $d.f. = 1$, $P = 0.95$; interaction n.s.). In addition, probability of female survival to the next year was not related to DEE or mass-specific DEE the year of treatment (DEE, $n = 66$: treatment, $\chi^2 = 2.10$, $d.f. = 1$, $P = 0.15$; year, $\chi^2 = 1.34$, $d.f. = 1$, $P = 0.25$; interaction n.s.; mass-specific DEE, $n = 66$: treatment, $\chi^2 = 1.11$, $d.f. = 1$, $P = 0.29$; year, $\chi^2 = 1.11$, $d.f. = 1$, $P = 0.29$; interaction n.s.). Probability of breeding the next year was not related to DEE or mass-specific DEE the year of treatment (DEE, $n = 51$: treatment, $\chi^2 = 0.21$, $d.f. = 1$, $P = 0.65$; year, $\chi^2 = 0.003$, $d.f. = 1$, $P = 0.96$; interaction n.s.; mass-specific DEE, $n = 51$: treatment, $\chi^2 = 0.93$, $d.f. = 1$, $P = 0.33$; year, $\chi^2 = 0.003$, $d.f. = 1$, $P = 0.96$; interaction n.s.).

Treatment in one year had no effect on a female's litter size at parturition, litter mass at parturition, or date of mating the next year (Table 11). Females gave birth on average 5 days later in 2011 than in 2010 and females were lighter at spring emergence from hibernation in 2011 than in 2010 regardless of treatment. Females raising augmented litters were lighter at spring emergence than females raising their natural litter size but were similar in mass to females raising reduced litters (Table 11).

DISCUSSION

We found that litter enlargement was metabolically costly as females raising augmented litters had significantly greater rates of energy expenditure at peak lactation than females in both the control and reduced groups (Table 10). A similar effect of litter size on metabolic rate has been documented in both experimentally manipulated and naturally occurring brood sizes of birds and mammals (Gabrielsen and Mehlum 1987, Kenagy et al. 1990, Deerenberg et al. 1995, Humphries and Boutin 2000). Elevated metabolic rates associated with greater reproductive demand were predicted to reduce energy stores in somatic tissues, ultimately resulting in reduced fecundity or survival. However, we found no direct relationship between daily energy expenditure or mass-specific energy expenditure on the probability of over-winter survival or subsequent breeding.

That increased metabolic rates associated with greater reproductive demand had no impact on survival is interesting because reproductive effort has been linked to reduced immune function (Deerenberg et al. 1997, Hanssen et al. 2005), susceptibility to disease or parasites (Festa-Bianchet 1989, Neuhaus 2003), oxidative stress (Alonso-Alvarez et al. 2004, Dowling and Simmons 2009), and impaired function of DNA repair mechanisms (Kirkwood 1990), all of which could increase probability of mortality. Whether or not these tradeoffs are a function of energy allocation is debatable (Zera and Harshman 2001, Harshman and Zera 2007). Nevertheless, the creation of reactive oxygen species is directly related to metabolism (Alonso-Alvarez et al. 2004), and animals likely cannot escape the oxidative stress associated with elevated reproductive effort. This suggests that Columbian ground squirrels are either capable of remediating potential detrimental effects of reactive oxygen species, that the buildup of reactive oxygen species over one or two reproductive seasons is not enough to incur irreparable cellular damage, or that over the course of the animal's lifetime greater reproductive effort would result in lower survival, all of which would be worthy of further investigation.

The only negative effect of current reproduction on the mother we found was on body mass. The year after litter manipulation, females raising augmented litters were lighter at spring emergence from hibernation than females in the control group (Table 11). This trend was also detected in a previous study of Columbian ground squirrels in which litter size was enlarged by one pup (Hare and Murie 1992). Interestingly, females raising augmented litters had similar body masses at litter weaning to females in control and reduced groups, but were lighter than control females at spring emergence from hibernation the next spring (Tables 10, 11). This suggests that females of augmented litters either lost more body mass or did not gain as much mass between weaning and hibernation or utilized more of their somatic energy reserves during hibernation than control females. However, this did not appear to have long-lasting repercussions as augmented females were able gain mass compensatorily between emergence the next spring and the start of lactation later that season, as indicated by the similar body sizes of females from all three treatment groups at parturition (Table 11).

Contrary to our predictions, litter augmentation and the resulting energetic cost did not result in higher mortality or reduced fecundity for females raising enlarged litters, supporting previous experimental and correlative studies that found no evidence of a cost of reproduction in this species (Hare and Murie 1992, Risch et al. 1995, Dobson et al. 1999). A short-term ecological cost to reproduction may not be evident in this species for several reasons. First, there may not be a tradeoff between current and future survival or reproduction. Tuomi et al. (1983) outlined several instances in which survival and fecundity need not be directly related to reproductive effort, such as when resources allocated to reproduction are utilized at a different rate than mobilization of somatic tissue stores or when reproductive demands are supported primarily by increasing energy intake rather than utilizing somatic fat stores, as is the case with income breeders. Columbian ground squirrels, like most other small mammals, are considered income breeders (Broussard et al. 2005). Although some female squirrels in our population lost mass during lactation, there was no difference in the frequency of females gaining or losing mass among treatment groups. Mass change during lactation may be better attributed to variation among females in territory quality, foraging efficiency, or reflect individual differences in changes in mammary mass. Evidence of a cost of reproduction in capital breeders such as bighorn sheep and red deer (Clutton-Brock et al. 1983, Festa-Bianchet et al. 1998b) and no tradeoff between current and future reproduction in several income breeders (Murie and Dobson 1987, Hare and Murie 1992, Mappes et al. 1995, Risch et al. 1995, Humphries and Boutin 2000) lend support to Tuomi's hypothesis. However, effects of current reproductive effort on maternal survival and subsequent fecundity have been detected in other small income breeders (Huber et al. 1999, Koivula et al. 2003)

Second, reproductive costs may only be incurred under certain stressful environmental conditions, such as low food availability or high population density (Bell 1986, Festa-Bianchet 1989). Although both years of litter manipulation in this study were mild years in terms of temperature and precipitation (personal observation), density was particularly high in our population. Density of our population was higher than the average calculated from other populations of Columbian ground squirrels (our population: 14 lactating females/ha; other populations: 9 lactating females/ha; Dobson 1990). Thus, if there are ultimate fitness costs to current reproductive investment, they should have been evident in our population.

Third, any cost of reproduction might be masked by maternal adjustment of litter size post manipulation. There is evidence that female Columbian ground squirrels adjust their reproductive effort to environmental cues by reducing litter size after birth (Murie et al. 1980). However, although litter loss occurred in our population there was no difference among treatment groups in the probability of whole or partial litter loss and females raising augmented litters produced more weanlings than females in control or reduced groups. These results contrast those of Neuhaus (2000), where females raising enlarged litters had fewer young survive to weaning. Although females in our study weaned more pups, those pups were smaller in size and grew at a slower rate than pups in control and reduced litters. This is consistent with previous records of a tradeoff between offspring number and quality in this species (Skibiel et al. 2009) and supports the proposition that a tradeoff between litter size and individual quality is more common than a tradeoff between current reproductive effort and fecundity or survival (Lindén and Møller 1989, Roff 1992).

It has been previously shown that smaller Columbian ground squirrel weanlings with slower growth rates have reduced survival over-winter (Skibiel et al. 2009). The population of the current study was no exception to this trend: the smaller, slower growing pups of augmented litters had a lower probability of survival through their first hibernation. These results contrast previous manipulative studies of litter size in Columbian ground squirrels and bank voles that showed no difference in offspring survival after independence among litters that were enlarged, reduced, or remained the same (Hare and Murie 1992, Oksanen et al. 2001, Koivula et al. 2003) but is consistent with a reduced number of surviving young after independence in red squirrels in augmented litters (Humphries and Boutin 2000). Our results suggest that in our population of Columbian ground squirrels, compensatory growth of smaller offspring raised in larger litters does not occur.

In conclusion, although females raising enlarged litters incur greater energetic costs during lactation, this does not appear to impact maternal survival or future breeding and does not support the cost of reproduction tradeoff. Instead, females supporting large litters allocate limited resources among more offspring, producing slower growing and smaller young with poorer prospects of survival post-independence. That females raising their natural litter size produce young with a greater probability of survival over females raising enlarged litters lends support to the hypothesis of individual optimization of reproductive effort and is consistent with prior empirical work on the evolution of litter size in Columbian ground squirrels (Risch et al. 1995). Although we were unable to address it in this study, it is possible that females incur a cost of reproduction in terms of lifetime reproductive potential. Future studies examining lifetime reproduction of females raising enlarged broods might provide further insight into the evolution of life history strategies.

Table 9. Effect of treatment in 2009 on offspring and maternal characteristics in 2010 for those females subjected to a litter size manipulation in 2009 and 2010 ($n = 30$).

Traits	d.f.	F-statistic	P-value
Offspring			
Mass at birth (g)	2, 24	0.19	0.83
Mass at weaning (g)	2, 18	1.77	0.20
Growth rate (g/day)	2, 18	2.13	0.15
Maternal			
Litter size at parturition	2, 24	1.06	0.36
Litter size after manipulation	2, 24	1.06	0.36
Litter size at weaning	2, 24	1.03	0.37
Parturition date	2, 24	0.19	0.77
Mass at parturition (g)	2, 24	0.16	0.86
Mass at weaning (g)	2, 19	0.14	0.87
Female mass change (g)	2, 19	0.08	0.93
DEE (kJ/day)	2, 19	0.37	0.69

Note: Two-way ANOVA including 2009 treatment and 2010 treatment as predictor variables and an interaction term. Treatment in 2010 was n.s. for all traits except litter size after manipulation. All interactions n.s. Female mass change is change in mass from birth to weaning. Offspring masses and growth rates are averages within litter. Growth rate is mass change from birth to weaning divided by lactation length in days. DEE = daily energy expenditure.

Table 10. Traits of females raising reduced, control, or augmented litters.

Trait	Reduced (R)	Control (C)	Augmented (A)	d.f.	F-statistic	P-value	Comparisons		
							C vs R	C vs A	R vs A
Mass at parturition (g)	505.0 ± 9.3 (19)	503.2 ± 7.6 (38)	505.0 ± 10.8 (19)	2, 72	0.02	0.98	n.s.	n.s.	n.s.
Parturition date	141.4 ± 1.3 (19)	143.3 ± 0.9 (39)	141.4 ± 1.3 (19)	2, 73	1.24	0.30	n.s.	n.s.	n.s.
Age	4.4 ± 0.4 (17)	5.0 ± 0.3 (36)	4.1 ± 0.5 (18)	2, 67	1.28	0.28	n.s.	n.s.	n.s.
Mass at weaning (g)	506.0 ± 9.6 (15)	500.4 ± 9.1 (28)	488.8 ± 14.8 (16)	2, 55	0.60	0.55	n.s.	n.s.	n.s.
DEE (kJ/day)	695.4 ± 30.7 (16)	753.8 ± 26.1 (33)	921.5 ± 48.8 (17)	2, 62	9.30	0.0003	n.s.	*	*
Mass-specific DEE (kJ/g/day)	1.37 ± 0.1 (16)	1.49 ± 0.1 (33)	1.82 ± 0.1 (17)	2, 62	9.25	0.0003	n.s.	*	*

Note: Two-way ANOVA including treatment and year as predictor variables and an interaction term. Parturition date and female mass at weaning also differed between years (see results). All interactions between year and treatment n.s. Means ± S.E. * = significant difference between treatment groups according to Tukey comparison of means. Partuition date is julian date of litter birth. DEE is daily energy expenditure. Sample size is included in parentheses.

Table 11. Breeding characteristics of females in the year following treatment

							Comparisons		
Trait	Reduced (R)	Control (C)	Augmented (A)	d.f.	F-statistic	P-value	C vs R	C vs A	R vs A
Litter size at parturition	3.1 ± 0.2 (15)	2.8 ± 0.1 (28)	3.0 ± 0.17 (12)	2, 51	0.65	0.53	n.s.	n.s.	n.s.
Litter mass at parturition	35.7 ± 2.2 (15)	32.8 ± 1.4 (28)	35.8 ± 2.4 (12)	2, 51	0.89	0.42	n.s.	n.s.	n.s.
Mating date†	121.9 ± 1.8 (15)	121.4 ± 1.5 (31)	126.1 ± 5.1 (13)	2, 55	1.11	0.34	n.s.	n.s.	n.s.
Spring emergence mass (g)†	386.0 ± 6.6 (15)	406.5 ± 9.3 (31)	363.8 ± 20.4 (13)	2, 55	3.5	0.04	n.s.	*	n.s.
Mass at parturition (g)	503.0 ± 8.1 (15)	513.6 ± 8.4 (28)	503.8 ± 11.3 (12)	2, 51	0.39	0.68	n.s.	n.s.	n.s.

Note: Two-way ANOVA including treatment and year as predictor variables and an interaction term. All interactions between year and treatment n.s. Mating date is the julian date when mating occurred. * = significant difference between treatment groups according to Tukey comparison of means. † denotes significant effect of year on the variable (mating date: year, $F_{1,55} = 5.30$, $P < 0.0001$; spring mass: year, $F_{1,55} = 7.47$, $P = 0.008$). Sample size is included in parentheses.

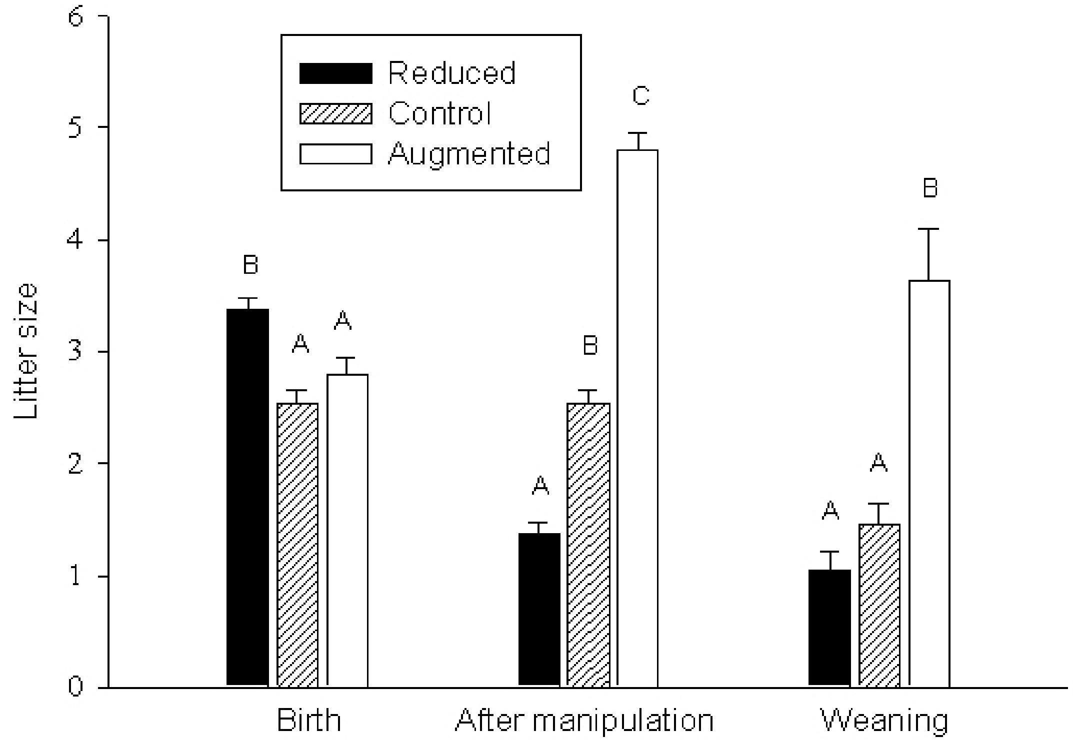

Figure 7. Comparison of litter size among treatment groups at three different time points: at birth, after litter size manipulation, and at weaning. The reduced group had two pups removed after birth, the augmented group had two pups added, and the control group had no change in litter size. Disparate letters indicate significant differences among treatment groups. Error bars represent standard errors. *N* = 39 control litters, *n* = 19 augmented litters and *n* = 19 reduced litters.

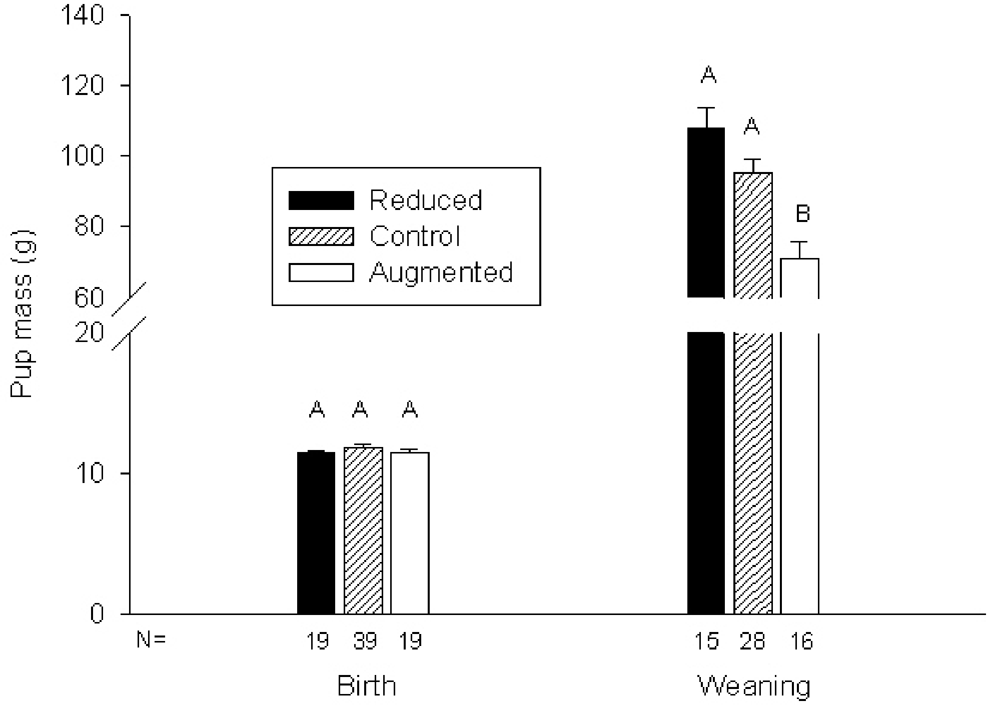

Figure 8. Comparison of average pup mass at birth and at weaning among treatment groups. The reduced group had two pups removed after birth, the augmented group had two pups added, and the control group had no change in litter size. Disparate letters indicate significant differences among treatment groups. Error bars represent standard errors.

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