Proximate composition of milk of captive nine-banded armadillos (*Dasypus novemcinctus*).

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Abstract

Armadillo dams have a potentially unique challenge in that their pups rapidly grow a bony carapace, suggesting a high requirement for the transfer of calcium and phosphorus from dam to pups via milk. We examined milk samples from 10 armadillo dams, samples collected at days 1 – 6, 14 – 15, 33 – 38, and 49 – 51 after birth. Water, protein, sugar, fat, ash, Ca and P content were assayed using standard methods at The Smithsonian National Zoological Park Nutrition Laboratory, and gross energy (GE) was calculated from protein, sugar and fat.

Introduction

Milk is the first and sole food of mammalian neonates. No matter what the adult diet, carnivorous, herbivorous, or omnivorous, all mammals start life as lactivores. Milk provides the nutrients for metabolism, growth, and development. The macronutrients in milk are water, fat, protein, sugar, and minerals such as calcium and phosphorus. Although these ingredients are common to all milks, the relative proportions can vary tremendously among species (Oftedal and Iverson, 1995; Skibiel et al., 2013). The composition of a species' milk provides insights into its evolution, ecology, patterns of growth and development, and many other aspects of life history.

The nine-banded armadillo, *Dasypus novemcinctus* (Mammalia, Xenarthra, Cingulata) is the only member of this South American superorder to expand its range into the United States. Armadillo dams have a potentially unique challenge in that their pups rapidly grow a bony carapace, suggesting a high requirement for the transfer of calcium and phosphorus from dam to pups via milk. The armadillo's carapace accounts for 16% of its total body weight and its ossification occurs mostly after birth (Vickaryous and Hall 2006, Anderson and Benirschke 1966).

The goals of this study were to characterize the nutrient composition of nine-banded armadillo milk across lactation and compare its milk composition with that of other mammals. Milk samples were collected at four time points during lactation from wild-captured female armadillos that gave birth in captivity, and the samples were assayed for proximate nutrient content (water, fat, protein, sugar, ash [total minerals], and calcium and phosphorus). We predicted that armadillo milk would have high concentrations of Ca and P, and, as Ca and P in milk is bound in micelles formed by casein proteins (Holt and Carver, 2012), a high protein content as well.

Materials & Methods

Ten adult female armadillos wild-captured in northwest Arkansas during the winters of 2008 and 2009 (n=7) and 2013 (n=3) birthed litters in the animal quarters of the University of the Ozarks. Nest boxes were checked morning and evening for newborn litters, which allowed for the birth date to be known within approximately +/-12 h; the day a litter was discovered was considered day 0 for days postpartum (pp).

Milk samples were collected during the first week postpartum (days 1 – 6), and at the ends of the second week (days 14 – 15), fifth week (days 33 – 38) and seventh week (days 49 – 51). Dams were anesthetized IM with ketamine/domitor cocktail in 2008, 2009 and isoflurane gas in 2013. Oxytocin was administered intravenously (0.25 ml/dose, 20 USP units/ml, repeated to effect). The milk samples were stored at -20 ^{0}C until analyzed at the Smithsonian National Zoological Park (SNZP) Nutrition Laboratory in summer 2013. Milk was collected from four of the dams at all time points, from 3 time points for one dam and two time points from another. The remaining four dams are represented by a single milk sample each collected between day 2 and 10 postpartum for a total of 25 milk samples.

Nutrient assays

All samples were assayed for water (dry matter), fat, total sugar, crude protein (CP), calcium and phosphorus using standard methods (Hood et al., 2009) at the SNZP Nutrition Laboratory; gross energy (GE) was calculated. Briefly, for water determination, milk samples were aliquoted, weighed, and dried in a forced convection drying oven for 3.5- 4 hours at 100ºC and then reweighed (AOAC, 1990). Total nitrogen (TN) was determined through a Dumas nitrogen gas analysis procedure using a carbon, hydrogen, and nitrogen elemental gas analyzer (Model 2400, Perkin Elmer, Norwalk, CT). Total nitrogen was multiplied by 6.38 to determine CP (Jones, 1931). Crude milk fat (total nonpolar lipid) was measured by a micro-Röse-Gottlieb procedure, which involves 3 sequential extractions with diethyl ether and petroleum ether following disruption of the milk fat globules with ammonium hydroxide and ethyl alcohol (Hood et al., 2009). Total sugar was analyzed by the phenol – sulphuric acid colorimetric procedure (Dubois et al., 1956; Marier and Boulet, 1959) using lactose monohydrate standards. Replicate sugar samples were read at 490 nm with a microplate reader and accompanying software (MRX TC Revelation, Dynex Technologies, Chantilly, VA). Results were multiplied by 0.95 to correct for water of crystallization in the standard.

Ash was determined by placing dried milk samples in a muffle furnace at 550°C for 8 hours. The ash was digested in nitric acid and perchloric acid on a hot plate within a perchloric acid-rated fume hood. The resultant acid digests were diluted with distilled deionized water. Calcium was measured using atomic absorption spectrophotometry (Model 800 Perkin Elmer Analyst Flame/Furnace Atomic Absorption Spectrophotometer, Perkin Elmer Co, Waltham, MA) at 422.7 nm using a nitrous oxide flame (AOAC, 1990). Phosphorus was determined by the AOAC-Modified Gomorri colorimetric method and read with a microplate reader and accompanying software (MRX TC Revelation, Dynex Technologies, Chantilly, VA) at 450 nm (AOAC, 1990; Gomorri, 1942).

Gross energy (kcal/g milk), or GE, was calculated using the formula: GE = (9.11 kcal/g * % fat + 5.86 kcal/g * % crude protein + 3.95 kcal/g * % sugar)/100. This equation has the potential to slightly overestimate gross energy because it fails to correct for non-protein nitrogen (Perrin, 1958; Oftedal, 1984). However, it has been verified against gross energy values measured by bomb calorimetry for milk from several species, including rhesus macaques and bongo (Hinde et al., 2009; Petzinger et al., 2014).

Statistical analysis

The composition of the 21 milk samples from the six dams with longitudinal samples was investigated using analysis of covariance, with dam as the categorical parameter and days postpartum as the covariate. The relationships among milk constituents was examined using Pearson correlation.

Results

Mean values for all milk constituents at each of the four collection time points from the 21 longitudinal samples from six dams are presented in Table 1. There was no difference among dams in the composition (protein, fat, sugar, and ash) of the longitudinal milk samples after accounting for days postpartum ($p > .29$ for all constituents); days postpartum was a significant factor for all these constituents ($p < .005$ for all constituents).

A characteristic of nine-banded armadillo milk is high concentrations of both protein and minerals (ash) throughout lactation. Protein concentration was the highest of the milk solids at all time points and ash values were higher than sugar values for the latter two time points (Table 1). Milk ash content rises consistently over lactation from about 1% of milk to above 3% by one month (Table 1). Calcium and phosphorus accounted for about half of the mineral content of the milks (Table 1). Both Ca (Figure 1) and P milk content were positively correlated with milk protein content ($r = 0.8$; $p < .001$ for both), and highly correlated with each other ($r = 0.99$, $p < .001$).

The GE content of armadillo milk is relatively high (Table 1), consistent with the high protein and moderate fat content. Armadillo milk GE increased over lactation ($r = 0.759$, $p < 0.001$), increasing from 0.912 kcal/g at days 3 – 6 to 1.44 kcal/g at days 49 – 51. Protein contributes most of the energy during the first two weeks of lactation (average 52% of GE, S.D.= 7.5), and does not change significantly with time. Fat energy increases rapidly over the lactation period and by 35 days pp contributes a similar percentage of energy as that provided by protein (33-51 days pp.- GE average: 46% from fat, S.D.=4.9, average 47% from protein, S.D.=4.3). Sugar contributes much less energy to milk than either protein or fat and its contribution decreases over time from an average of 12% (S.D.=4.8) to 6.9% (S.D.=0.94).

Discussion

Armadillo milk is unusual in the high proportion of energy from protein and its high mineral content. These two properties of armadillo milk are likely functionally connected. Armadillo pups have an unusual growth challenge in that they must grow the bony plates that serve as armadillo armor. The high mineral content of armadillo milk provides the pups the necessary minerals to grow their bony carapace. Calcium and phosphate in milk is predominantly bound within casein micelles (Holt and Carver, 2012). A milk high in calcium and phosphorous by necessity will be high in casein protein. Armadillo milk calcium content is strongly associated with milk protein content (Figure 3), suggesting a high ratio of casein proteins to whey proteins. The high protein content of armadillo milk may be required in order to transfer the appropriate amounts of calcium and phosphorus from mother to offspring via milk for the growth of the bony plates of the carapace.

The high proportion of potential metabolizable energy in armadillo milk from protein strongly suggests that armadillo pups metabolize amino acids for metabolic energy. The digestion of the casein proteins releases the calcium and phosphorous, but also provides a large amount of amino acids for metabolism. Armadillo milk protein content exceeds 10% during late lactation, and probably provides a substantial excess of amino acids relative to the growth requirement. Casein proteins are not balanced, containing a large proportion of proline, a non-essential amino acid, and being deficient in the sulphur amino acids. The excess amino acids from casein protein digestion likely will be catabolized for metabolic energy.

A reliance on milk protein for energy may be a feature of Xenarthran lactation. There is very limited data on milk from any of the Xenarthrans, but what there is suggests that protein is higher in concentration than either fat or sugar. Giant anteater (*Myrmecophaga tridactyla*) milk is also relatively high in protein, providing a higher percentage of Ge than armadillo milk (61% of the GE; M. Power unpublished data).

Conclusions

We suggest that the evolution of armadillo bony plates required a milk relatively high in protein content. Specifically, we predict the armadillo milk protein to be high in casein proteins to carry the necessary Ca and P to form the bony plate. We also predict that armadillo pups will utilize milk protein for metabolizable energy in addition to depositing it into tissue for growth.

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Table 1. Mean values ± SEM for armadillo milk constituents at four time points across lactation.

	Pup age 3 – 6 days	Pup age 14 – 15 days	Pup age 33 – 38 days	Pup age 49 – 51 days
N	5	6	5	5
DM (%)	16.8 ± 2.2	20.0 ± 1.0	24.9 ± 1.4	24.7 ± 0.5
GE (kcal/g)	0.912 ± .142	1.105 ± 0.080	1.394 ± 0.029	1.439 ± 0.029
Protein (%)	8.0 ± 1.2	9.1 ± 0.3	11.0 ± 0.4	11.1 ± 0.4
Fat (%)	3.6 ± 0.9	5.0 ± 0.7	7.2 ± 0.1	7.6 ± 0.3
Sugar (%)	3.0 ± 0.2	3.0 ± 0.2	2.4 ± 0.1	2.3 ± 0.1
Ash (%)	1.6 ± 0.3	2.3 ± 0.2	3.4 ± 0.1	3.6 ± 0.1
Ca (%)	0.41 ± 0.12	0.70 ± 0.07	1.13 ± 0.04	1.17 ± 0.06
P (%)	0.26 ± 0.06	0.42 ± 0.04	0.62 ± 0.02	0.65 ± 0.03

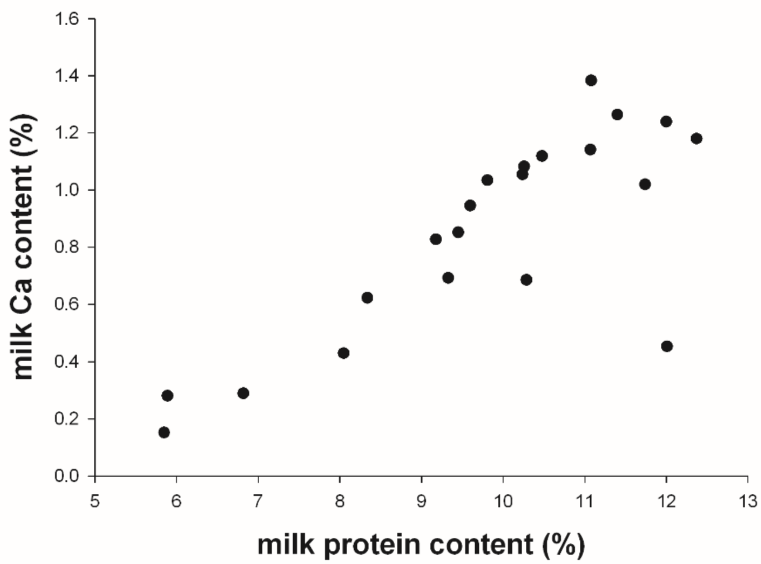

Figure 1. Armadillo milk Ca concentration is highly correlated with milk protein concentration.