ABSTRACT

WAGNER, JENNA. Milk Macronutrient Comparisons Among Domesticated and Wild Perissodactyla Species. (Under the direction of Dr. Kimberly Ange-van Heugten).

To study differences among milk macronutrients, archived and current perissodactyla samples from 8 domestic horses (*Equus caballus*), 13 rhinoceroses (representing three species: *Diceros bicornis, Ceratotherium simum*, and *Rhinoceros unicornis*), 5 tapirs (representing two species: *Tapirus bairdii*, *Tapirus indicus*), and 16 wild equids (representing six species: *Equus ferus przewalskii*, *Equus grevyi*, *Equus quagga, Equus zebra, Equus africanus somaliensis*, *Equus asinus*) were inventoried and analyzed from varying institutions (n = 16). Depending on the individual, milk samples were selected between 3 and 200 days post parturition (dpp). Milk samples were analyzed at the Smithsonian National Zoo and Conservation Biology Institute (SZCBI) nutrition laboratory for major macronutrients: crude protein (CP), dry matter (DM), fat, sugar, and gross energy (GE). Gross energy was calculated with the formula: GE = (9.11 kcal/g * % fat + 5.86 kcal/g * % CP + 3.95 kcal/g * % sugar)/100. Statistical analysis was conducted with R software and RStudio to compare macronutritional values among species and families. Analysis of covariance (ANCOVA), analysis of variance (ANOVA), and linear regression models were conducted along with Tukey post – hoc assays when ANCOVA and ANOVA statistical significance were present. Research study one assessed the accuracy of milk macronutrient microquantity analyses with non-bovine milks at the SZCBI compared to literary microquantity analysis values at commercial labs. This test used domestic mares' milk in comparison to previous mare milk published values. This study also examined how individuality and geographic locations influenced the milk macronutritional composition in mares' milk. Using mares as a model, the study confirmed that microquantity macronutritional analysis at SZCBI was accurate in calculating macronutritional values for non-bovine / perissodactyla

milks, as the study values were within literature ranges. The mares in the studies were housed in two separate academic institutions that were located on separate coasts of the United States, North Carolina (NC) State University (Raleigh, NC) and California (CA) Polytechnic State University (San Luis Obispo, CA). The mare milk from NC had higher CP and lower fat concentrations than the CA mares (ANCOVA, $p < 0.05$). The difference in milk CP and fat concentrations may be linked to the variation in diets among the two institutions. When assessing the macronutritional composition variation among individual mares, there was limited evidence to support that individuality impacts the milk macronutritional concentrations. The second study was to analyze perissodactyla species milks with microquantity macronutritional analysis from numerous individuals that were housed in various locations (n=16). There were limited differences among species within each of the three perissodactyla families (Equidae, Rhinocerotidae, Tapiridae) apart from the white and black rhino, with the black rhino having higher CP, fat, and GE concentrations than the white rhino. The difference between these rhino species may be indicative of their natural different dietary habits, with white rhinos being grazers, while black rhinos are browsers. When comparing among the perissodactyla families, large differences were observed in CP content among Tapiridae and Equidae milk (Tapir=5.93%, Domestic horse CP=2.14%). There were also trends of macronutritional composition changes over lactation in tapirs and rhinos, where sugar was an increasing macronutrient for both. These findings indicate that equine milk is an insufficient substitute for tapirs. This study has a positive informational impact on emerging perissodactyla breeding and conservation programs with strong demand for accurate milk formulas and replacements.

Milk Macronutrient Comparisons Among Domesticated and Wild Perissodactyla Species.

by
Jenna Corinne Pastel Wagner

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APPROVED BY:

Dr. Kimberly Ange -van Heugten Committee chair	Dr. Shweta Trivedi Committee Co-chair
Dr. Larry J. Minter	Dr. Michael Power

DEDICATION

I would like to dedicate this thesis to my husband and our daughter.

BIOGRAPHY

Jenna Wagner was born in Silver Spring, Maryland. Jenna attended college at St. Mary's College of Maryland where she received her B.S. in Biology and Environmental Studies with a minor in Mathematics. She unearthed her career's passion for comparative animal nutrition (specifically exotic milk comparisons) when she was an intern with the Smithsonian Nutrition Laboratory. She was able to leverage that experience to return to the Smithsonian as a full time Research Assistant. In addition, she had the opportunity to expand her knowledge and experience with the nutrition team at San Diego Global as a visiting fellow. Jenna has happily settled in Raleigh, North Carolina with her husband. Her goals are to shape this developing field, play a vital role in saving animals from extinction, and develop the next wave of scientists through mentorship, teaching, and publications.

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Chapter 1: Literature Review

Introduction

This is a comprehensive literature review of the milk and evolutionary history of the perissodactyla order. In addition, this review provides the general descriptions, diet, gestation / rearing, and comprehensive milk nutritional composition literature on domestic horses, wild equids, rhinoceroses, and tapirs.

A defining characteristic used to classify a mammal is the ability to produce milk.[1] Milk provides all essential nutrient to a newborn mammal. However, there has been little research done on composition of milk across the whole mammalian phylogeny.[2] When studying the composition of milk, the bulk of the research has been conducted on bovine (cow) and human milk.[3]

Although milk is an evolutionary and nutritionally important substance, there are many factors that can limit a mother's ability to provide suckling. The mother sometimes will not produce enough milk, will reject the neonate, or the neonate / mother can be sick resulting in them to be separated.[4] Since the neonate still cannot ingest solids and needs nutrients that would be found in the milk, there are formulas developed to replicate milk.[5] These milk formulas have been used in animal breeding and conservation programs although species milk suitability concerns exist.[6] Conservation programs attempt to increase or sustain a species population through habitat conservation, habitat restoration, public education, and breeding etc. For example, breeding programs within the Association of Zoos and Aquariums (AZA) Taxonomic Advisory Groups (TAG) and Species Survival Plan (SSP), are inter-institutional programs for threatened species to improve genetic diversity and population size.[7]

A species is normally determined to be at risk by a respected conservation organization, such as The International Union for Conservation of Nature (IUCN). The purpose of the IUCN is to track species population statuses and to aid conservation policy and efforts by identifying species that need targeted recovery efforts.[11] The IUCN Red List is an information source that tracks factors that can impact global biodiversity, and the threats that species are facing.[8]

Currently, a taxonomic class, the perissodactyla's, have 13 of their 17 species listed as vulnerable, endangered, or critically endangered; the remaining 4 are considered of least concern or near threatened.[8] The 3 taxonomic families in the perissodactyla order are: Equidae (equids), Tapiridae (tapirs), and Rhinocerotidae (rhinos).[1] Currently there are AZA taxonomic advisory groups that encompass all three of the perissodactyla families.[7] However, interestingly, the tapir group is associated within the same AZA taxonomy group as hippos (Hippopotamus), peccary (Tayassuidae), and pigs (Sus), not with their closest taxon (relatives), the equids, or rhinos.[7]

For each perissodactyla family, there is a developed AZA Care Manual.[9] The care manuals provide suggested habitat design, enrichment, veterinary care, training, diet, and reproductive information including milk replacer formulas. The milk formulation recommendations for wild equids, such as zebras, are commercial equine milk replacers.[9] The milk formulation recommendations for rhino milks also recommends an equine milk replacer.[9] Other institutions, if equine replacer is unavailable, have developed rhino milk replacers with bovine milk replacers.[10] The current AZA milk formula recommendations for all tapir species are goat milk although a few tapir holding institutions utilize equine replacers,[11] which shows that there are preference discrepancies for tapir milk replacer. There is limited data on the milk nutritional composition of perissodactyla species excluding the domestic horse (*Equus caballus*) to be able to base milk formulations off of.[12] Feeding incorrect milk replacers can result in malnutrition, developmental problems, and even death for neonates[13].

For threatened species, it is especially optimal to breed and rear healthy individuals so that they can sustainably contribute to the population. The current lack of milk replacer data among species indicates a need for further research on the perissodactyla milk composition and determination of proper formulation among species and among different lactation stages (and parities).

Milk

Milk production is an evolutionary trait that developed in order to allow synapsid vertebrates to thrive on land when there were limited water resources.[6] The mammary gland evolved to allow efficient secretion of the nutritional fluid (milk). The mammary glands, which evolved from apocrine glands[14], are a system developed by alveolar nodules and extensive adipose tissue, that with the proper hormonal triggers, initiate lactation.[15]

Milk is excreted and formed after gestation and can occur in late gestation (pre-parturition).[16] Milk is the sole nutritional source for neonatal mammals in early development stages.[6] There are multiple stages of lactation with their own vital roles to healthy neonatal development. The milk stages are: pre–partum milk production, colostrum, early lactation, mid-lactation, and late lactation.[6] Pre-partum milk is mammary secretions before parturition, it is similar to colostrum and usually occurs in small quantities.[16] After birth, for the next 24 to 72 hours, colostrum is produced. Colostrum is a special type of milk that is denser nutritionally and contains a high concentration of immunoglobulins.[17] Early lactation also sometimes referred to as transitional milk, is colostrum transitioning to milk by having a higher water content and lower nutritional content. Mid lactation, or peak lactation is what is often referred to as the classic "milk", which is a white opaque liquid.

The primary ingredient in milk is water.[2] This coincides with the evolutionary theory behind milk, which was to provide young water, thus allowing the animals to exist further from major water sources.[14] Milk is then further comprised of sugars, fats, proteins, vitamins, and minerals.[6]

The most common sugar in milk is lactose which is a disaccharide that is glucose and galactose bonded together.[20] There are other carbohydrates found in milk, called oligosaccharides.[21] Fats are a major component of milk composition. In domestic equine milk, triglycerides are most of the fat, representing 80 - 85%.[22] However, other fats from cholesterol, phospholipids and free fatty acids consist of approximately 19.5 %.[22] The fat globule molecules in equine milk are relatively small compared to bovine and human milk.[23] The smaller fat molecule size effectively cause a greater efficiency on lipid metabolism.[23] The common proteins in milk are whey and casein proteins.[24] The proteins are used not just for nutrients, but also as packaging for minerals so that they do not bond and solidify in the mammary glands.[25] Casein protein along with fat separate from milk.[25] Whey protein is digested faster than casein and is often utilized for muscle growth.[25] There are a large assortment of essential vitamins and minerals found in milk.[20]

In milk chemistry, there is an inherent osmosis ratio, that in result, limits the nutritional composition of milks.[26] In a milk with the majority milk macronutrient being sugar, or a high sugar milk, will result in high water content. This means that milk cannot biochemically be high sugar and high fat. There is also a negative correlation between the concentration of protein to the concentration of sugar present in a milk.[27] This is important to consider when developing milk formulas and distinguishing differences in species milk nutritional composition.

Sugar, fat, and protein accumulate together and total the approximate overall gross energy or caloric energy found in milk.[28] Although the primary role of milk is to provide nutrients for a neonate, there is evidence of other important non-nutritional factors in milk. Milk contains immunoglobulins,[29] microbes,[30] hormones,[31] and growth factors.[23] There are numerous factors that can influence milk composition, including diet[32], phylogeny[2], time post parturition[33], location[34], and the gender of the baby.[35]

Perissodactyla Order

To perform a cohesive comparative study of the perissodactyla order, the phylogenetic identifiers and taxonomy should be examined.

Within the mammalia class, there are four placental mammal superorders (a taxonomic category), Afrotheria, Xenarthra, Euarchontoglires, and Laurasiatheria[36]. The superorder Laurasiatheria, is one of the most expansive and diverse superorders[37]. The Laurasiatheria superorder was identified by molecular and genetic sequencing and has since been repeatedly confirmed by multiple studies[38]. The superorder consists of interordinal phylogenetic comparisons and shared characteristics are unclear. There is a hypothesis that the linking characteristics of originating in Laurasia (hence the name) was also the root of the multiple order's evolutionary adaptations. Laurasiatheria contains five orders: Cetartiodactyla, Carnivora, Chiroptera, Eulipotyphla, and Perissodactyla. Reclassifications are evidence of the fluidity of taxonomic identifications, and phylogenetic studies. There have been debates and research studies to solidify and identify superorders, and suborder positions. Many of the orders within Laurasiatheria are currently under scrutiny within the scientific community.[39,40]

The phylogenetic position of perissodactyla is hotly debated with modern molecular and genetic studies[41]. Morphologically, there is a generally minute difference of perissodactyla from fellow Cetartiodactyla due to being odd–toed ungulates versus being even–toed ungulates.[42] However, there are studies that propose that these two orders should be combined in a clade "Feruungulata"[43]. Currently, however, perissodactyla is identified as an individual order characterized by being odd-toed ungulates, and since grouped this way they are often fed this way and despite controversy it is important to investigate this way.[44,45]

All species identified as perissodactyla are hindgut fermenters.[46] Hindgut fermenters are monogastric herbivores that have large cecum's that house symbiotic bacteria that aid in the digestion of cellulose.[47] The cecum is located after the small intestine hence the phrase "hindgut" since the digestion of cellulose occurs by fermentation in the latter part of the digestive tract.[48]

The perissodactyla order is an ancient order that dominated the Eocene period and originated in India.[44] In the Oligocene period artiodactyla order were competitors that successfully outcompeted the perissodactyla order.[48] This resulted in an ecological change / competition that resulted in the taxonomic order diversity to decline. Currently, there are 3 families which encompass 17 total species in the perissodactyla order. The families are Tapiridae, Rhinocerotidae, and Equidae.[50] The most common species in the perissodactyla order is the domestic horse (*Equus caballus*).

Domestic Horse

The domestic horse has been archaeologically documented since the early Neolithic period (7,000 BCE).[51] Since then, there have been over 300 different horse breeds developed by humans.[51] The American Quarter Horse is the most popular breed in the USA by comprising 39.5% of the horse population.[51] They grow to be 56 – 64 in tall.[51] The domestic horse has been utilized for transport [52], meat[53], and sport/companionship.[54] Even though there has been an increase of horse meat usage, in the United States, many people feel uncomfortable eating horse meat.[55] There are currently 7.25 million horses owned in the USA alone.[55] The domestic horse has a strong cultural presence as a companion and work animal.[56]

With millions of horses to care for, there is a need for husbandry care plans, including diet plans. The global horse feed industry alone in 2022 was a $7.58 billion business.[57] There are many diet formulations dependent on the age, breed, and work amount of the horse.[58] The nutritional standards for horse diets are in the National Research Council (NRC) Nutrient Requirements of Horses.[58]

Table 1.1 is the recommended nutritional NRC concentrations for domestic horses. These nutritional requirements can be accomplished with a diet formulation that includes an array of commercial pelleted diets in accompaniment with hay/forage. It is recommended that a horse is fed 2% of its body weight in hay.[58]

Table 1.1. The NRC General Nutrient Requirements for Domestic Horses at growing mature, and pregnant/lactating life stages. These are nutrients that are of major focus to the NRC for horse nutritional requirements with the exception of water intake.[58]

Nutrient	**Growing**	**Mature/ Maintenance**	**Pregnant/ Lactating**
Dig. Energy (mcal/kg)	2.45 -2.90	2.00	2.25 - 2.60
Crude Protein (%)	12 - 15	8	10 -13
Calcium (%)	0.6	0.3	0.4
Phosphorus (%)	0.3	0.2	0.3
Magnesium (%)	0.1	0.1	0.1
Potassium (%)	0.3	0.3	0.4
Selenium (mg/kg)	0.1	0.1	0.1
Vitamin A (IU/kg)	2000	2000	3000
Vitamin D (IU/kg)	800	300	600
Vitamin E (IU/kg)	80	50	80

Gestation lasts for 11 - 12 months on average for the domestic horse.[59] It is challenging to determine the natural rearing behavior of domestic horses since there is often human intervention.[60] Foals start weaning or are weaned at 4-6 months,[62,63] although, weaning in 'feral' horses occurs at 8 – 9 months.[61]

Often, when breeding horses, there is a need for supplemental milk.[62] There are commercial horse milk formulas that are available for public purchase. The formulas are normally a dry matter powder with instructions for various dilutions depending on the age of the foal (Buckeye Nutrition, Dalton, OH; Land O Lakes, Arden Hills, MN; Sav-A-Caf, Chilton, WI; Tribute Equine Nutrition, Sandusky, OH). Colostrum formulas are also available for horse foals.[63]

Table 1.2. Example of a popular horse milk formula (Land O Lakes, Mare's Match Foal Milk Replacer, Arden Hills, MN) mixing and feeding instructions.

Mixing Directions
Use the plastic cup provided to measure the milk replacer powder. Each cup holds about 0.25 pound of powder. Always weigh Land O Lakes® Mare's Match® Foal Milk Replacer powder for accurate mixing. Use only low sodium (<50 ppm) water for mixing and feeding! Mix 0.25 pound dry weight of Land O Lakes® Mare's Match® in 1 quart (2 pints) of 110-120°F water by slowly adding powder to water while stirring. Feeding temperature should be 100-105°F.
Feeding Guidelines
Use the following schedule as a guideline for feeding reconstituted milk replacer:

Age of Foal	**Number Feedings Per Day**	**Feeding Rates (Quarts) Per Foal Daily***
1 day	-	colostrum
2-7 days	4	4-8
2nd Week	4	6-12
3rd & 4th Week	3	8-15
5th Week	3	6-12

6th-8th Week	2	4-8

The average nutritional composition of mare's milk is 10.3% dry matter, 2.1% crude protein, 1.2% fat, 6.4% lactose, and 0.4% ash, with the gross energy (kcal/kg) being 480.[64] A longitudinal study was conducted on mare milk macronutrient composition with 14 mares from Iowa State University Horse Barn, the samples were sent to Dairy Lab Services in Dubuque, IA for fat, protein and lactose analysis. Dairy Lab Services analyzes milk with macro spectrometry equipment standardized for cow milks.[65] The study found that protein decreased over 12 weeks, while fat and lactose percentages remained relatively consistent over the 12 week time period.[65]

Wild Equids

Wild equids, for the sake of this review, are any equids that are not domesticated horses. The current extant species of wild equids are: Przewalski's horse (*Equus ferus przewalskii*), Somali Wild Ass (*Equus africanus somaliensis*), Onager (*Equus hemionus*), Kiang (*Equus kiang*), Grevy's Zebra (*Equus grevyi*), Mountain Zebra (*Equus zebra*), and Plains Zebra (*Equus quagga*).[48]

Zebras are a species most identified by their distinct black and white stripes. They range in the eastern and southern plains of Africa. Plains Zebras are IUCN listed as near threatened, Mountain Zebras as vulnerable, and Grevy's Zebras as endangered.[66–68] All zebras range on the African continent. The Grevy's zebra range on Kenya and Ethiopia.[68] Plain zebras' range on the grassland or savanna of southeast Africa.[66] The Mountain zebra range on the mountains of south Angola and Namibia.[67]

Somali wild ass, Onager, and Kiang are all wild asses. Somali wild asses are IUCN listed as critically endangered, Onager as endangered, and Kiang are of least concern.[69–71] The Somali wild ass ranges in the desert and shrublands of northeastern Ethiopia and Eritrea.[70] Onager range in southwestern Mongolia primarily.[70] The Kiang resides in the Plateau of Tibet and are more domesticated compared to the other wild equids.[71]

Przewalski horses are the last remaining wild horses. They are virtually extinct in the wild, although reintroduction programs have been established.[72] The Przewalski horse was previously known to range through Mongolia and China.[73]

All wild equids are grazers. Grevy's zebras have been noted to consume more bulk compared to their ruminant competitors .[74] The plains zebra, select areas with the greatest amount of grass despite quality.[75] Although their habitat is more limited due to altitude, mountain zebras select areas due to high grass coverage.[76] Somali wild asses live in more arid areas and vegetation is sparse requiring greater migrations to meet nutritional requirements and are also where domestic donkeys descend from.[77] Recommended dietary requirements according to the Association of Zoos and Aquariums (AZA) Husbandry Manual for wild equids, are based on modified domestic horse diets.[9] The only overarching modification for all wild equids is the availability of multiple meals and feed diversity.[9]

Gestation in Grevy's zebra lasts for 390 days.[78] In comparison, mountain and plains zebras have a gestation period of 365 days.[79] The Somali wild ass gestation period is 377 – 404 days.[80] Przewalski horse gestation period lasts for 330 – 340 days.[81] Transfers of zebra and Przewalski horse embryos into domestic mares has been successful.[82] This proves promising for the ability to expand breeding programs with domestic surrogates.[83] Which also begs to question if milk from the domestic surrogate is the best fit for the foal.

Grevy's zebra foals suckling behavior is different from other equids, where they have longer bouts of suckling. They suckle for the same amount of time as a 5-month-old wild domestic horse when they are a 6 week old foal.[84] A cause for this suckling behavior is theorized to be due to the arid climate.[84] Comparatively, mountain zebras suckle for the longest period of time, then plains, then Grevy's zebra, then the domestic horse (suckles 1 – 2 times per hour for 3 minutes).[85]

Oftedal and Jenness[86] conducted a wild equid milk composition study with microquantity analysis methods. Sugar, was analyzed by the picric acid method,[87] fat was determined with the Babcock volumetric method and a modified Roese-Gottlieb method.[88] The nitrogen (used to determine crude protein content) was determined by calculating the total nitrogen by direct Nesslerization and micro-Kjeldahl digestion [89] then, filtering the non-protein nitrogen.[14] They analyzed samples from 5 mountain zebra females, 5 plains zebra females, and 5 Przewalski horse females.[87] All samples were from the San Diego Zoo. [14] The data indicates that all the wild equids milk where similar to horses, a high sugar milk ranging between 6 – 7% sugar (carbohydrates).

Table 1.3. Oftedal and Jenness 1988, nutritional composition summary of wild equids including percentage of total solids (i.e., dry matter), fat, protein, sugars, and ash.

Paper	Species	Total Solids	Fat	Protein	Carbohydrates	Ash
Oftedal and Jenness, 1988	Onager	12.00%	3.67%	2.75%	6.52%	0.35%
Oftedal and Jenness, 1988	Mountain Zebra	10.00%	1.02%	1.56%	6.92%	0.32%
Oftedal and Jenness, 1988	Plains Zebra	11.30%	2.20%	1.63%	7.00%	0.38%
Oftedal and Jenness, 1988	Przewalski Horse	10.50%	1.50%	1.55%	6.72%	0.33%

Rhinocerotidae

Rhinos are megaherbivores. Rhinos have 3 toes on their front and back limbs.[90] Rhinos have horn(s) on their nose. The horns on some species of rhino's averages to 90cm long. The horn is made of keratin and is a buildup of keratin on the outer dermis of the rhino snout.[91] The horn can grow back if cut or damaged.[92]

There are 5 species of Rhino: White (*Ceratotherium simum*), Black (*Diceros bicornis*), Greater – One Horned (*Rhinoceros unicornis*), Sumatran (*Dicerorhinus sumatrensis*), and Javan (*Rhinoceros sondaicus*).[93] There are subspecies of rhinos including, the northern white rhino, southern white rhino, southern black rhino, eastern black rhino, and western black rhino. All of these can be distinguished by their regional habitat, physiological, and genetic properties.[93] For this specific review detail of the distinct subspecies will not be discussed although it is important to note that there are breeding opportunities between subspecies that may aid in the future conservation efforts.

The white rhino is the most common rhino species and is listed as near threatened on the IUCN Red List.[94] The northern white rhino is functionally extinct with the last male passing away in 2018.[95] The southern white rhino ranges primarily in Namibia, Zimbabwe, Kenya, and South Africa.[94] White rhinos can be characterized by their lack of a distinct single prehensile lip and ‘square lips’.[94]

The black rhino is a critically endangered species according to the IUCN Red List.[96] There are 3 subspecies of black rhino (the 4th, western black rhino, went extinct in 2011). The black rhino’s range is primarily south of the Democratic Republic of the Congo.[96]

The greater one horned rhino is the largest of the rhino species and the IUCN Red Lists identifies them as vulnerable.[97] They live in northeast India and Nepal. They are more uniquely identifiable from the other rhinos from its “armored” look and single horn.[97]

The Sumatran rhino is IUCN listed as critically endangered with only 30 known individuals existing.[98] They range in in the Sumatran and Borneo islands. The habitat is primarily forest. The Sumatran rhino is the smallest of the rhino species and appears hairier than its relatives.[99]

The Javan rhino also is a IUCN species listed as critically endangered.[100] The remaining individuals live in a national park in Java, Indonesia. Also called the lesser one-horned rhino, the Javan rhino resembles the greater one-horned rhino with the similarly “armored – skin” and single small horn.[101]

The diet is distinctly different among rhino species depending on if they are grazers or browsers. White rhinos are grazers and play a specific role in the ecosystem by uniquely grazing short grass communities that cannot be replaced by other grazers in the habitat.[102] Black rhinos on the other hand are browsers, with grasses comprising of only 4.5% of their diet from fecal analysis.[103] Greater one horned rhinos are classified as grazers with 70 to 89% of their diet consisting of grass species.[104] Diet formulations are based on NRC horse recommendations for all rhinoceros species.[9,105]

White rhinos gestate for 17 months.[106] Black rhinos gestate for 15 months with five-month trimesters.[107] Greater one horned rhinos’ gestation period is on average 16 months.[108]

There was an interesting behavioral pattern observed in white rhinos when rearing different gendered calves.[109] The study indicated that male calves suckled more frequently and had delayed weaning, compared to female calf counterparts. There was also a response to male calves based on their vocalization, to allow more nursing sessions, whereas vocalization did not appear to impact mother’s reactions to their daughters. This study was from 14 radio-tracked white rhinos in a game park in South Africa.[109]

There are studies on the milk macronutritional composition of some species of rhinos (Table 1.3). Greater one horned rhino milk samples were sampled from two females at Zoo Basel, Switzerland who were on the same diet and analyzed with microquantities, with the exception of the sugar assay. From female A, only 3 samples were taken at 0, 5, and 10 dpp.

There was more extensive sampling of female B with there being 15 unique sample dates (0, 8, 20, 32, 41, 79, 109, 141, 165, 199, 229, 313, 363, 385, 404 dpp). Ashing was conducted in a muffled furnace, fat was determined by a Soxhlet fat extraction with EE and PE,[110] CP was estimated by the Kjeldahl method,[111] lactose concentrations were determined with a Mid-IR Spectroscopy that was calibrated with cow milk.[112] Aschaffenburg et. al. used the same microquantity methods that Gregory et. al. did, to analyze milk macronutrients of a black rhino.[113] They analyzed a single captive black rhino milk sample from 19 months post parturition (~ 570 dpp).[113] Aschaffenburg and Gregory et. al. analyzed the same female milk from separate parturitions (~ 10 years apart). Both used similar methods and their result averages were not identical, indicating a slight discrepancy in milk macronutrient composition among parities. Free-ranging white rhinoceroses' milks were analyzed from 15 females in the Northern Cape province of South Africa using microquantity analysis methods.[12] The dry matter and ash were calculated by drying in a forced convection oven and then incinerated at 550°C for 2 hours. The crude protein was assayed by a nitrogen analyzer and subtracting the non-protein nitrogen by crude protein, then multiplying the nitrogen content be 6.38. Fat was extracted with the Folch method. Only saccharides were extracted, using liquid chromatography.[12]

Table 1.4. A literature summary of total solids i.e. dry matter, fat, protein, sugar and ash concentrations of various rhino species milk averages from their corresponding published studies[15,112–116]

Paper	Species	Total Solids	Fat	Protein	Sugar	Ash
Aschaffenburg et. al., 1961	Black Rhino	8.10%	Trace	1.54%	6.06%	0.34%
Gregory et. al., 1965	Black Rhino	8.82%	0.20%	1.40%	6.62%	NR
Gimmel et. al., 2018	Greater One Horned Rhino	8.44%	3.04%	16%	7.46%	3.80%
Zainal, et. al. 1998	Sumatran Rhino	7.52%	0.07%	1.37%	5.38%	NR
Matthews, 1973	White Rhino	14.23%	1.73%	7.34%	NR	NR
Osthoff et. al., 2020	White Rhino	9.37%	1.76%	0.93%	7.93%	0.40%

NR = Not Reported

Tapiridae

Tapirs are in appearance, pig-like, with large, rounded bodies and four small legs. They have a unique prehensile snout that is around 7 in in length.[117] Tapirs are unique to other odd toed ungulates as they have an even 4 toes on their front limbs and 3 toes on their back limbs.[117] Tapirs all have unusual colorations of their fur pattern dependent on the species. The infant fur color is different than adults, with unique stripes and dotting resembling a watermelon.[48] The research on tapirs has been limited as they are relatively reclusive.[117] Tapirs are semi-aquatic, spending most of their time browsing, copulating, and resting in bodies of water.[117]

There are 4 species of Tapir: Malayan (*Tapirus indicus*), Lowland (*Tapirus terrestris*), Mountain (*Tapirus pinchaque*), and Baird (*Tapirus bairdii*). There was a hypothesized fifth species of tapir noted in 2013, a genetic test was also performed revealing differences between the new “Kabomani” tapir and the lowland tapir.[118] Local Brazilians have identified the subspecies as a separate species as well.[119] However, with genetic and morphological follow–up studies, it has been concluded that there is not a sufficient distinction between the Kabomani and lowland tapirs.[120,121] Neither the Tapir Specialist group nor the IUCN Red List have declared this 5th species, in concerns that the evidence is “limited”.[8]

The Malayan tapir is unique to other Tapiridae species by being the only tapir located in the eastern hemisphere. The regional habitat of the Malayan tapir is in Malaysia, and to Southern most parts of Thailand and Myanmar. The Malayan tapir is endangered under the IUCN Red List.[122] Malayan tapirs have a distinct black and white fur coloration with the front half and legs having black fur, and the dorsal down to the butt having white fur. Malayan tapir habitats are neotropical and are dense.[123] The lowland tapir is the most abundant species of tapir[124]. The lowland tapir is classified as vulnerable in the IUCN Red List.[124] The lowland tapir has a crest type mane on the top of its head that runs down to the top of the shoulder blades.[125] The Baird's tapir is identified as endangered on the IUCN Red List.[126] They have dark, relatively monochrome fur.[127] The mountain tapir is the smallest of the tapir family and the most endangered according to the IUCN Red List. The mountain tapir is also referred to as a woolly tapir due to its distinctive "woolly" fur.

In general, there is still very little knowledge of general tapir ecology.[129] Tapirs are understood to be a browsing herbivore. Mainly living in neotropical environments, they use their prehensile snouts to support browsing beneath and around the canopy ground.[130]

A food selection trial was performed with Malayan tapirs in the Sungai Dusun Wildlife Reserve (Selangor, Malaysia).[131] The reserve is a dense tropical secondary forest environment that allows for a widespread selection of species for browsing. Tapirs browsed 217 species of the 1142 species available in the reserve, however, sixty percent of all the plants selected for consumption were only 30 species.[131] They favored new growth and small saplings.[131] An intake trial was conducted on captive Baird's tapirs (n=22) in UK zoological collection.[132] The intakes of digestible energy were calculated assuming that digestion of food nutrients would coincide with the standard equation used for domestic horses. Seventeen of the tapirs exceeded the assumed maintenance requirements of 0.6 kJ/Digestible Energy (DE) /kg 0.75/d.[132] Body condition scores and fecal scores were positively correlated to dry matter intake and digestible energy intake.[132]

There is no conclusive evidence that nutrient requirements differ greatly among tapir species, however the diet for the Malayan tapir is proportionally larger due to their larger size.[9] Provided below (Table 1.5) is an interpretation of the nutrient requirements provided by the AZA Tapir Care Manual, which was heavily based on known nutrients requirements for horses.[58]

Table 1.5 Dietary target nutritional concentrations (90% DM basis) of diet for any tapir species.

Nutrient	**Concentration**
Crude protein (%)	14 - 18
Calcium (%)	0.20 - 0.65
Phosphorus (%)	0.15–0.34
Magnesium (%)	0.07–0.10
Potassium (%)	0.27–0.38
Selenium (mg/kg)	0.09
Vitamin A (IU/kg)	1000–3500
Vitamin D (IU/kg)	200–500
Vitamin E (IU/kg)	120–350

Commonly, tapirs only deliver one calf per pregnancy.[133] Baird tapir gestation averages 392 days[134], lowland tapir gestation averages 394 days [135], and Malayan tapir gestation averages 399 days.[136] Overall, gestations ranged between 13 – 14 months. There may be sporadic foraging and supplemental browsing within a week post parturition, however nursing will occur for 6 to 8 months post parturition.[137] The mother will be with it's young for 1 – 2 years.[133]

Very little research has been done on tapir milk. An assessment of lowland tapir milk was analyzed in 2014 of six total females using both macro and microquantity analysis methods.[138] Three females had samples taken longitudinally and there appears to be no duplication within a time period. Total and whey protein composition were determined by using the Lowry method[139], casein was calculated by subtracting the total from the whey quantity. Carbohydrates were measured with the Winzler procedure.[140] Fat was extracted by the Folch's procedure, then followed by a chemical process to perform fatty acid analysis. Perinn's formula was used to calculate gross energy.[138] A summary of a lowland tapir milk analysis was performed in 1965 and methodology nor dpp were reported. The milk compositions were demonstrated to be a relatively high protein high sugar milk.[138] Methodology for other tapir milk studies were not reported.

Table 1.6. Literature summary of total solids i.e. dry matter, fat, protein, sugar and ash concentrations averages for tapir milks.[138,141,142]

Paper	Species	Total Solids	Fat	Protein	Sugar	Ash
Ormrod, 1967	Lowland Tapir	15.67%	3.40%	5.70%	5.61%	0.96%
Toyoda et. al., 1970	Lowland Tapir	Not Reported	4.10%	5.90%	5.30%	1.00%
Van Niewenhawie et. al., 2014	Lowland Tapir	Not Reported	2.47%	6.34%	5.41%	NR

NR = Not reported

Discussion

To compare perissodactyla milk composition, habitats, diets, gestation periods, and rearing habits should also be examined.

The regional habitats of perissodactyla species broadly vary among plains, tropical forests, and mountains. Three tapir species are geographically in the New World. Residing in the New World, compared to the Old World (like the rest of the perissodactyla species), can influence phylogenetic traits due to evolutionary seperation.[143,144] The different habitats can also influence dietary and energy requirements based on water availability and energy regulation.

The diets of perissodactyla species are all similarly dependent on their ability to ferment with their large cecum. As they are all herbivores, there is a distinction between the grazers and the browsers. The distinction between grazing and browsing is on a spectrum, and not necessarily a binary scale. Seven of the seventeen species of perissodactyla are identified as browsers. This includes the entire Tapiridae family, black rhino, Sumatran rhino, and Javan rhino. None of the equids would be considered to have a browser dependent diet. Despite these differences in diet types, the current diet recommendations from the AZA for these species are all based on the NRC nutritional requirements for horses.[9,145]

Since scientists formulate diets for the perissodactyla species based on the closest domestic relative, milk replacers are similarly formulated. Just like the diets, can it be truly assumed that the nutritional requirements of a perissodactyla neonate is the same as a domestic foal? The current literature on milk nutritional composition of perissodactyla comparisons, are limited due to sparse literature of perissodactyla milks and to low n values overall from lack of available individuals. From the limited literature it could be hypothesized that there is a difference among the tapir milk from the rest of the perissodactyla families. The fat and crude protein content may be increased in tapir milk, as the sugar content is lower than the rest of the perissodactyla families. Using the Perrin formula with the literature averages, it may also be hypothesized that all tapir species have a higher energy milk than other perissodactyla species.

There is a clinical application and need to understand the composition of perissodactyla milks since breeding programs exist for all the perissodactyla species. There are taxonomical advisory groups for rhinos, tapirs (hippo, peccary, pig & tapir TAG), and equids. Specifically, there are efforts to conserve and breed for white rhinos, black rhinos, greater one-horned rhinos, Grevy's zebra, Hartmann's Mountain zebra, plain's zebra, Asian wild horse (Przewalski horses), African wild ass, Asian wild ass, Baird's tapir, and Malayan tapir.[7] To develop the most accurate milk replacers for at risk species, their milk nutritional composition needs to be further investigated. However, there are limitations to accessing exotic animal milks. The most obvious is that the exotic animals are not domesticated, therefore they are harder to handle and milk. In addition, the overall population size (n) of the species is already lower than domestic species, but there is additionally a low access and permit hinderances to the lactating perissodactyla population. These setbacks mean that the quantity of milk samples and quantity of milk per sample can be as low as 1ml from one day for a female's entire lactation period. This requires the need for microassays of milk that can be standardized or compatible with a variety of nutritional compositions. Due to these limitations, this study will (1) compare the accuracy of microquantity milk macronutrient analysis and (2) assess if individuality or location greatly impact concluding assessments of total milk macronutrient concentrations.

Due to the large data deficits in the nutritional composition of milk macronutrients (including fat, sugar, crude protein, minerals (represented as ash), and gross energy) in the perissodactyla order species throughout all stages of lactation, the goals of this current masters research project were to:

1) Confirm that microquantity analysis methods do not vary from traditional methods used in large dairy science organizations by comparing macroquanity analysis literature from domestic horse milks macronutrient values to microsampled horse milk analyses.
2) Compare horse milk macronutrient data from the same breed and parity in two different regions of the United States (California and North Carolina) across lactation periods, to confirm reproducibility and possible milk macro nutritional composition variability within species.
3) Compare historical and current milk sample macronutrient data among perissodactyla species and taxonomic families over early and mid-lactation (excluding colostrum), to determine potential differences vital for neonates.

Conclusion

Milk is an evolutionary characteristic of the Mammalian class. Mammals need milk in their first stages of life, and it is imperative to healthy growth and development. Conservation and breeding programs for perissodactyla species base their supplemental milk replacer formulas on the closest domestic relative which is the horse with the exception of tapirs.

Chapter 2: Domestic Horse Milk Macronutritional Composition Analysis with Micro Quantities from Two Transcontinental States

Abstract

Quarter horse milk from the North Carolina State University (NC) (mares n=4) and California Polytechnic State University (CA) (mares n=4) equine centers was compared to investigate differences in macronutritional composition over 3 and 160 days after parturition. All mares were healthy and single parity. Major macronutrients were measured at the Smithsonian National Zoo and Conservation Biology Institute (SZCBI) by microquantity analysis of crude protein (CP), dry matter (DM), fat, sugar, and gross energy (GE). Few differences among individuals were observed; however Californian mares had greater CP and fat concentration means on a dry matter basis than the North Carolinian mares add P value used for detection. These findings indicate that diet or other environmental factors may play a role in macronutrient composition, however the macro nutritional means for all eight mares were within macroquantity value ranges found in previous literature. When comparing microquantity assay values to macroquantity values, they were found to be within normal range. This study supports the future use of microquantity analysis to threatened species, even when there is variations in geographical locations and individuals. Which will aid in clinical and scientific applications of milks for exotic species.

Introduction

The horse (*Equus caballus)* was domesticated as early as 7,000 BCE[49], and since then, over 300 horse breeds[50] have been used in transportation,[52] recreation,[54] and food industries[53] worldwide. Research on the nutritional composition of horse milk has been needed for two budding industries: (1) human consumption and (2) foal milk replacers. Mare milk has become a popularized ingredient in health beverages for human consumption leading to an increase in mare milk production in recent years.[146] Certain factors have helped spur this trend including the production of cheese from mares' milk[147] and the reduced likelihood of allergic reactions humans can have to horse milk relative to traditional cow's milk.[148] Mares' milk has also been researched for foal milk replacers, in case of orphanage, sickness, or general need of supplementation.[4] Equine milk formulas are commercially available for over-the-counter purchase and attempt to closely replicate a horse milk nutritionally throughout the different phases of lactation.[63] Milk composition can vary over time, but can also vary depending on the individual females and geographic location of the mare.

The average reported macronutritional composition of mare's milk is 10.3% dry matter (DM), 2.1% crude protein (CP), 1.2% fat, 6.4% lactose, 0.4% ash, and 480 kcal/kg energy(GE).[64] A longitudinal study was conducted on mare milk macronutrient composition with 14 mares from Iowa State University Horse Barn (Ames, IA) via Dairy Lab Services (Dubuque, IA) for fat, CP, and lactose analysis using equipment standardized for cow milks (*Bos taurus*).[65] The study found that CP decreased over 12 weeks post parturition, while fat and lactose percentages remained relatively consistent over the 12 week time period.[65] The analysis of mare's milk can be conducted with a variety of methods including large scale milk analysis companies. These companies analyze milk samples effectively but often require a minimum 20 ml of milk to be analyzed for each sample.[149] In addition, many milk labs are calibrated by cow milk standards due to cow's large presence in the milk industry.[150] Mare's milk varies in macronutrient composition in comparison with cow's milk.[151] Mare's milk has a lower fat, CP, ash, and caloric concentration. The sugar concentration in mare's milk is also higher than cow's milk (DM = 12.8 , CP = 3.5 , sugar = 4.9 , fat = 3.7, ash = 0.72, GE = 674 kcal/kg).[151]

When researchers investigate the macronutrient composition of non-bovine milks and especially threatened species' milks, the sample quantities are often small due to the nature of the collection opportunities and procedures.[2] At the world's largest exotic animal milk repository at the Smithsonian National Zoo and Conservation Biology Institute (SZCBI) (Washington D.C.), many of the archived samples were taken opportunistically.[152] Compared to a dairy farm, where approximately 8 gallons of milk[153] can be retrieved from one female cow a day through machine pumping, the act of milking an exotic animal presents many more limitations. In addition to the sample quantity being lower, there is also a concern with analysis that the laboratory standard curve range for macronutrient measurements encapsulates bovine milks. With this, standard curves cannot encompass the natural variation of macronutrient compositions present in other species that may contain macronutrient percentages outside of the bovine standard curve.

With microquantity analysis methods, 2ml of milk total is required. In addition, some of the measurements are not dependent on a standard curve, disregarding concerns with using bovine milk standards.[154] For the sugar analysis, lactose monohydrate standards are used, however the milk DM concentrations can be altered (dilutions with distilled water) to keep the measurements within the standard curves.[154,155] Although there are various methods to analyze milk macronutrients with micro and macro quantities, many agricultural departments use spectroscopy analysis which requires macroquantities due to ease and cost.[156] There are established standards of cow milk nutritional composition that has inherently made cow milk the default for milk method testing, however, milk macronutritional compositions have not been tested among non – bovine animals.[157]

Horse milk is an excellent model milk to assess the consistencies in milk macronutritional concentrations that could be impacted by bovine standards. The goal of this study was to (1) assess if common milk macronutrient composition analyses used for microquantity analyses is variable compared to larger labs using macroquantities and (2) to determine if there is significant variation in horse macronutrient composition depending on individual females and geographical location (North Carolina (NC) vs California (CA)). The aim of this study was to pursue milk macronutrient composition using microquantities analyses from domesticated species as a model for exotic species and to interpret statistical significance within a species despite potential differences in location and individual females.

Materials and Methods

Test Subjects

Quarter horse (*Equus caballus*) milk samples were acquired from eight single parity healthy mares. Four mares (NC1, NC2, NC3, NC4) (age=5–14; BCS=6–6.5) from the first geographic location were sampled at the NC State University equine research unit in Raleigh, NC. The mares were managed and cared for by the equine unit staff and volunteers. The mares were bred for educational and research purposes. They were not part of any treatment group. The mare's diets ranged from receiving 6-10 lbs of Purina Impact All Stages 12:6 Pellet (Purina, Arden Hills, MN) per day. They also received approximately 6 lbs of alfalfa (*Medicago sativa*) a day and had access to free choice oat (*Avena sativa*) hay. The mares were housed in covered barns and were provided 24/7 access to pasture.

The four mares (CA1, CA2, CA3, CA4) (parity = 1) from the second geographic location were sampled at the California Polytechnic State University equine center, San Luis Obispo, CA. The mares were managed and cared for by the equine unit staff and volunteers, bred for educational and research purposes and not part of any treatment group. The horses received 2 flakes of alfalfa hay, 1.5 quarts of proprietary in-house formulated grain mix (San Luis Obispo, CA) and 1.5 quarts of Platinum Performance Equine (Platinum Performance, Buellton, CA) feed in the morning, followed by 1 flake of grass midday, and then given 2 flakes of alfalfa and 1.5 quarts of proprietary in-house formulated grain mix (San Luis Obispo, CA) in the evening. These horses were housed in covered barns. The pasture access was limited to half days a week before foaling, for 3 – 5 days post parturition the mares were kept in the stalls, followed by 7 days of given access for 50% of the days, then returning to 24/7 access to pasture after 10 – 12 days post parturition.

Milk Collection

Before milk expression, the nipple area was cleaned with clean water and then dried. Manual expression was done by applying pressure to the area surrounding the nipple and squeezing firmly towards the nipple.[159] The milk was collected into clean Thermo Fisher Scientific (Waltham, MA) 2ml cryovials. Milk / colostrum samples were taken from the mares every day up until 7 days after parturition. After day 7, the milk samples were taken every 3 days. 4 weeks after parturition, milk sampling was done every 7 days. This continued until approximately 160 days post parturition (dpp). The average animal foaled between February 2022 - April 2022. The final collection was in August 2022.

Nutritional Analysis

The milk was frozen at approximately –28°C and until the end of collection and then overnight Fed Ex transported to SZCBI with dry ice or ice packs in insulated packaging. Milk macro nutritional composition analyses were completed in duplicates for DM, fat, CP, total sugar, GE, and ash at the SZBI Nutrition Laboratory (Washington, DC) following the lab’s standard protocols with duplicates.[155] Crude Protein (CP) was determined by multiplying total nitrogen by the conversion factor 6.38.[159] Total nitrogen was measured from an elemental analyzer (Model 2400; Perkin Elmer, Waltham, MA). Fat content was measured with a modified microfat Rose-Gottlieb procedure.[154] Total sugars were measured through a phenol-sulphuric acid colorimetric procedure[155,160] and using lactose monohydrate standards and read at 490 nm on a microplate reader (Model ELX808; BioTek, Winooski, VT). Ash was determined by placing dried milk in a muffle furnace at 550°C for 8 h. Dry matter was both measured and calculated throughout sample analysis when drying the milks while subsampling for CP analysis and ash at 100°C in a forced-air drying oven. A Gross energy (GE) formula verified against GE values measured by bomb calorimetry for milk from several species[161] was utilized for this study. The GE (kcal/g milk) was calculated using the formula: GE = (9.11 kcal/g * % fat + 5.86 kcal/g * % CP + 3.95 kcal/g * % sugar)/100.

Statistical Analysis

Macronutrient concentrations were assessed for GE, DM, CP, sugar, fat, and ash between 3 and 160 dpp. Although CA samples were collected maximum 130dpp, there was no statistical impact when including 5 NC samples from dpp > 130; in addition the samples allowed a more cohesive regression analysis for NC samples. The general concentrations of macronutrients were assessed on a milk basis, dry matter basis (DMB), and gross energy basis (GEB). Analysis was performed using R statistical software (The R Foundation, RStudio, Boston, MA), and statistical results of means, medians, and ranges were found for individual mares and housing locations.

Analysis of covariance (ANCOVA) was conducted among individuals and between locations for each of the assayed macronutrients. A post hoc Tukey's honest significant difference test was conducted if proceeding ANCOVA results we significant. Values were considered significant if the p value < 0.05. Multilinear regression models and Pearson correlations were determined to assess the relationship of macronutrient compositions over time.

Results

When comparing individual mares, there were 28 unique combination comparisons that were made among them which was taken into consideration when assessing the proportion of differences among individuals. When comparing locations, there were an even number of individuals for each group, representing two locations; California (CA) and North Carolina (NC). All foals were healthy and there were no abnormal nursing behaviors.

Table 2.1. Samples analyzed for each individual mare represented over days post parturition (DPP) timeline. The average amount of milk samples per mare = 11 (total n = 90).

Individuals	**dpp**	**n**
CA1	4 - 119	11
CA2	4 - 125	11
CA3	4 - 112	10
CA4	4 - 126	11
NC1	4 - 154	13
NC2	4 - 137	11
NC3	4 - 152	13
NC4	3 -128	10

CA = mares from California Polytechnic State University
NC = mares from North Carolina State University

When comparing milk dry matter percentage (DM%) among individual females, there was only a difference between NC4 and CA2, with CA2 having 2% greater DM (ANCOVA, Tukey, $p = 0.02$) (Figure 2.2). When comparing DM% between locations, there was no significant difference (ANCOVA, Tukey, $p = 0.08$) (Figure 2.3). The change of DM% over time (dpp) was insignificant, but decreasing (linear regression, R2 = 0.18, Pearson coefficient = -0.43). The average DM% was 11.04%.

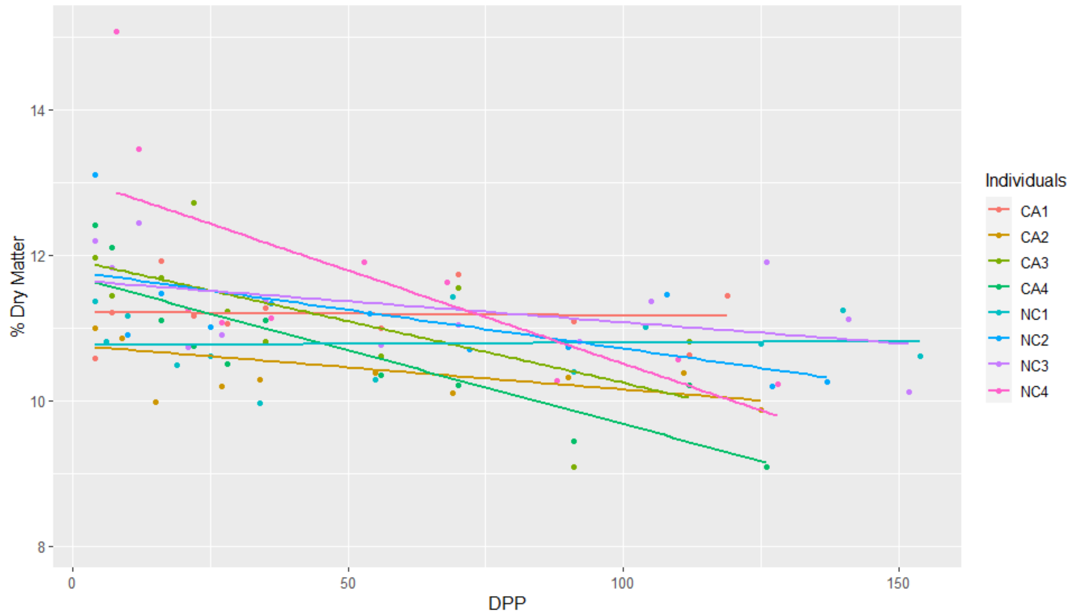


Figure 2.1. The trendlines and values of horse milk percent dry matter concentrations among eight individual horses for up to 160 days post parturition (DPP) at two housing locations (CA= 4 & NC= 4) (n = 90 milk samples, ANCOVA, p < 0.05).

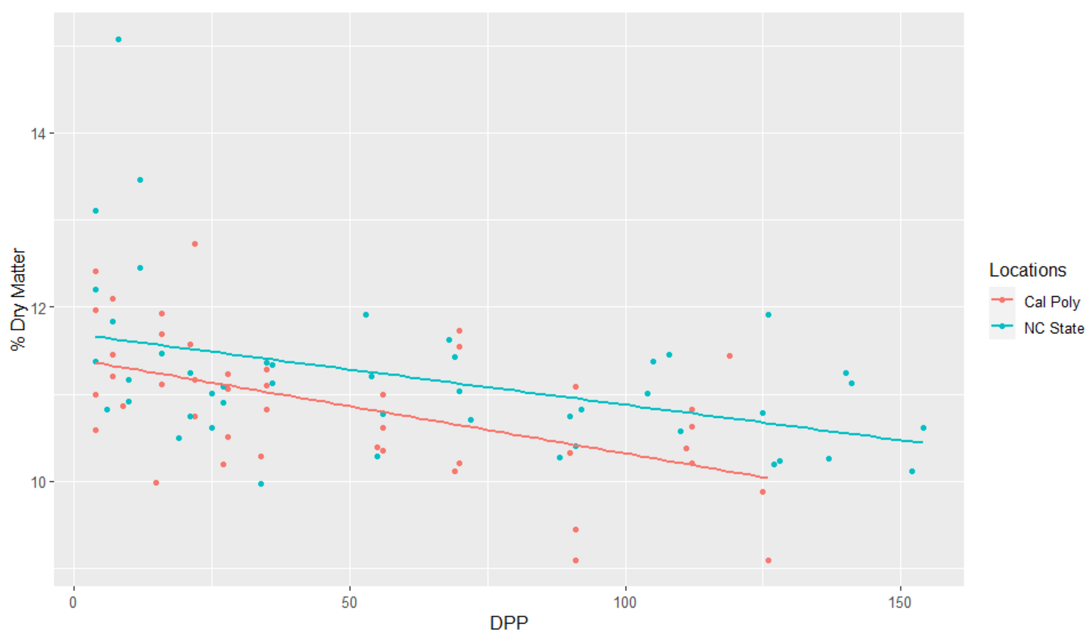


Figure 2.2. *Percent dry matter concentration trendlines of California Polytechnic State University (Cal Poly) and North Carolina State University (NC State) mares' milk over day post parturition (DPP) with associated regression lines (CA = 4, NC = 4) (n = 90, ANCOVA, p > 0.05).*

There were no differences in GE among individuals (ANCOVA, p = 0.26) nor location (ANCOVA, p = 0.058) (Figure 2.4 & Figure 2.5). There was no significant correlation between GE and DPP (linear regression, R^2= 0.16, Pearson coefficient = -0.42). The average GE was 49.70 kcal/g.

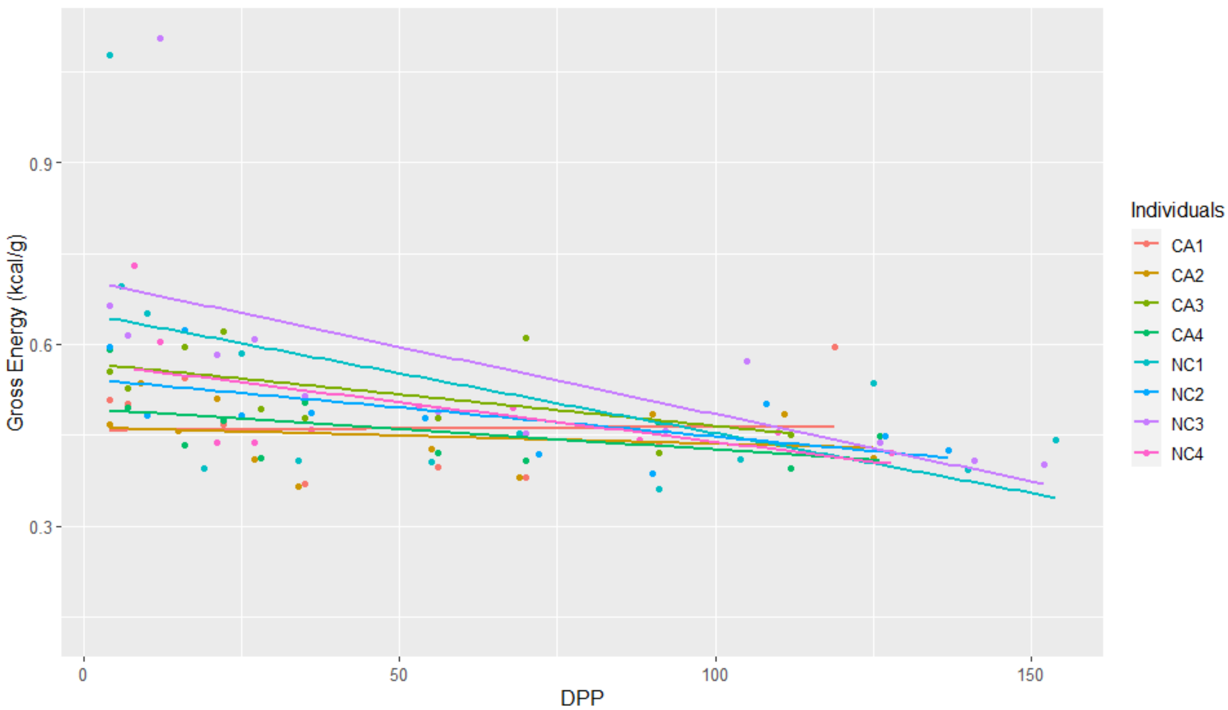


Figure 2.3. The trendlines and values of milk gross energy (kcal/g) concentrations among eight individual horses for up to 160 days post parturition (DPP) at two housing locations (CA= 4 & NC= 4) (n = 90 milk samples) (ANCOVA, p > 0.05).

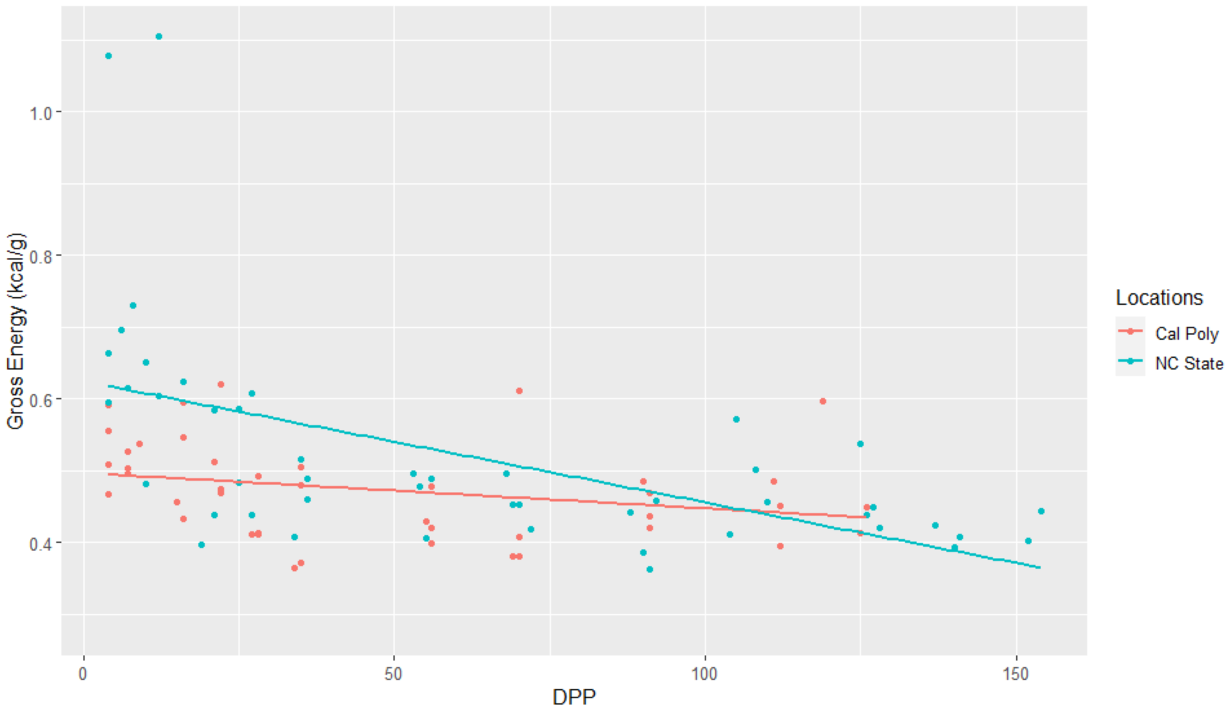


Figure 2.4. Gross energy (kcal/g) of California Polytechnic State University (Cal Poly) and North Carolina State University (NC State) mares' milk over day post parturition (DPP) with associated regression lines (CA = 4, NC = 4) (n = 90 milk samples, ANCOVA, p > 0.05).

The range for ash concentrations was between 0.00% - 7.02 DMB, with the average =3.11% DMB (0.40% of total milk). Ash concentrations were relatively constant over time on a DMB (linear regression, R2 = 0.19, Pearson coefficient = -0.45). There were no differences in ash concentrations (DMB) based on location (ANCOVA, p = 0.20) nor individuals (ANCOVA, p = 0.12) (Figure 2.6 & Figure 2.7).

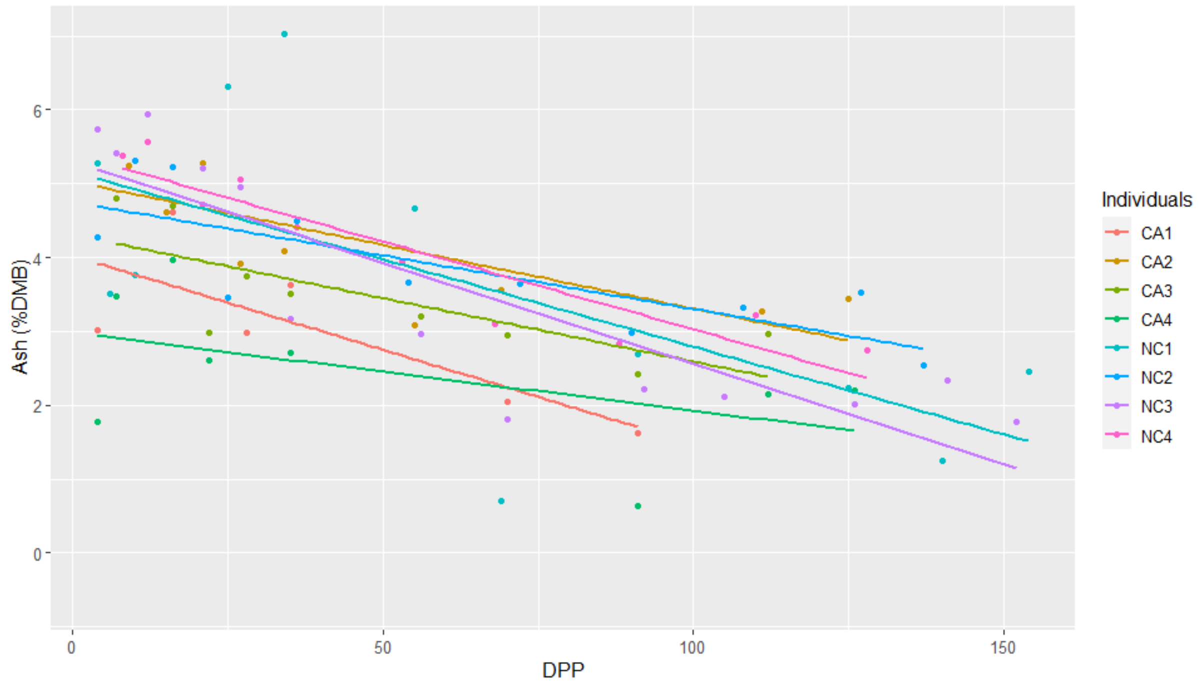


Figure 2.5. The trendlines and values of milk ash concentrations (dry matter basis) among eight individual horses for up to 160 over day post parturition (DPP) at two housing locations (CA= 4 & NC= 4) (n = 90 milk samples, ANCOVA, p > 0.05).

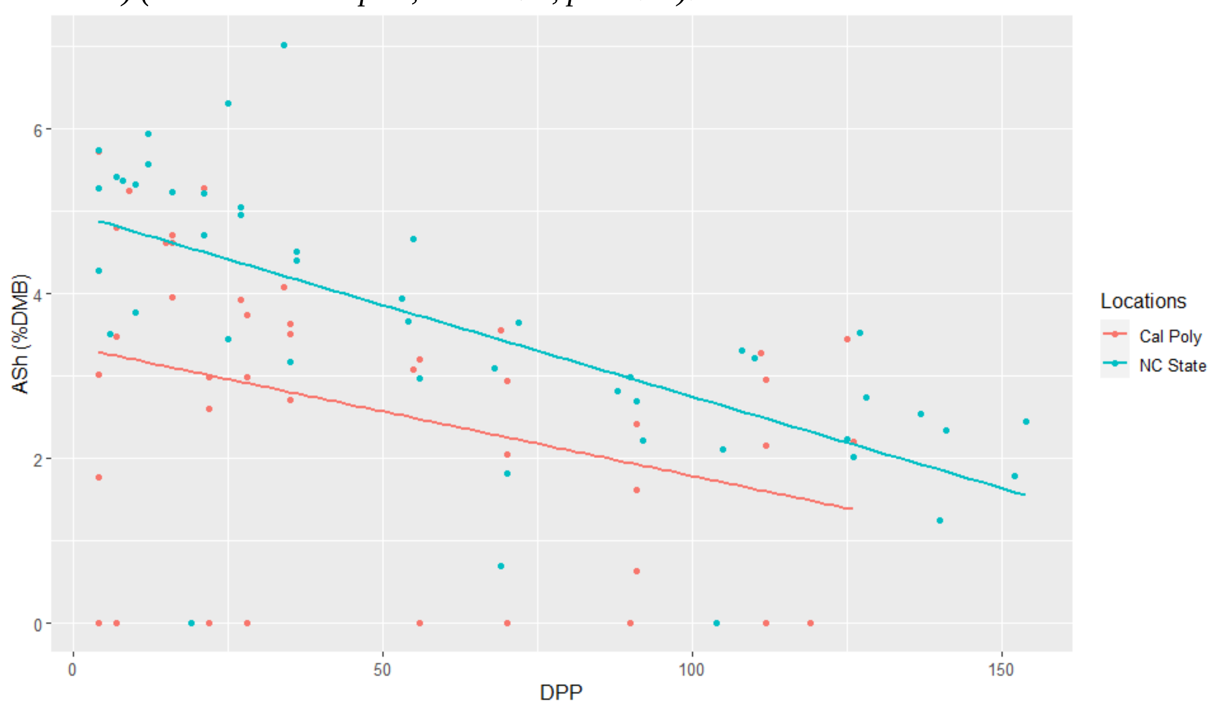


Figure 2.6. Percent ash concentrations (dry matter basis) of California Polytechnic State University (Cal Poly) and North Carolina State University (NC State) mares' milk over day post parturition (DPP) with associated regression lines (CA = 4, NC = 4) locations (n = 90 milk samples, ANCOVA, p > 0.05).

The range for fat concentrations was between 2.65 % and 20.09% DMB, with the average = 10.61% DMB and 1.18% on a total milk basis. Fat concentrations did not have a significant change over time on a DMB (linear regression, R2 = 0.12, Pearson coefficient = -0.35). Cal Poly mares had significantly higher milk fat concentrations (DMB) than NC State mares (ANCOVA, $p < 0.001$) (Cal Poly = 12.24 % DMB, NC State = 9.10 % DMB) (Figure 2.9). There were 4 differences in fat concentrations (DMB) among individual comparisons which were all interinstitutional; between NC2 < CA3 ($p = 0.005$), NC4 < CA3 ($p = 0.01$), NC2 < CA1 ($p < 0.001$), NC4 < CA1 ($p = 0.003$) (Figure 2.8).

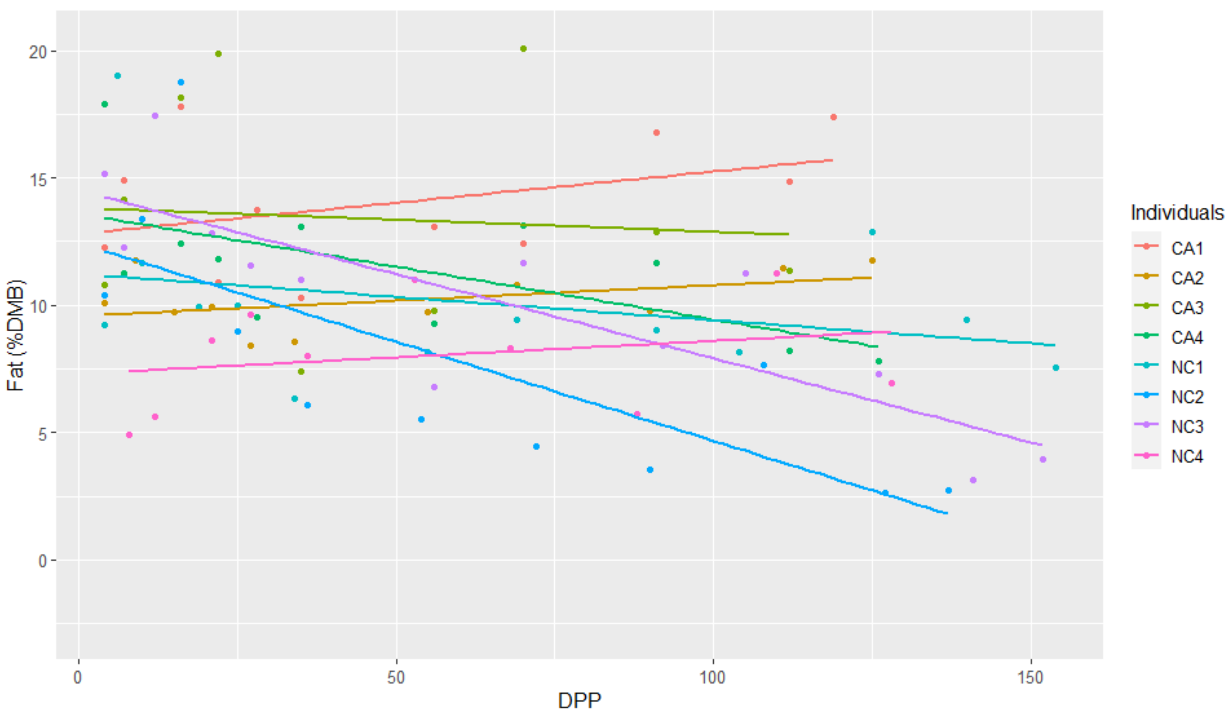


Figure 2.7. The trendlines and values of milk percent fat concentrations (dry matter basis) among eight individual horses for up to 160 over day post parturition (DPP) at two housing locations (CA= 4 & NC= 4) (n = 90 milk samples, ANCOVA, p < 0.05).

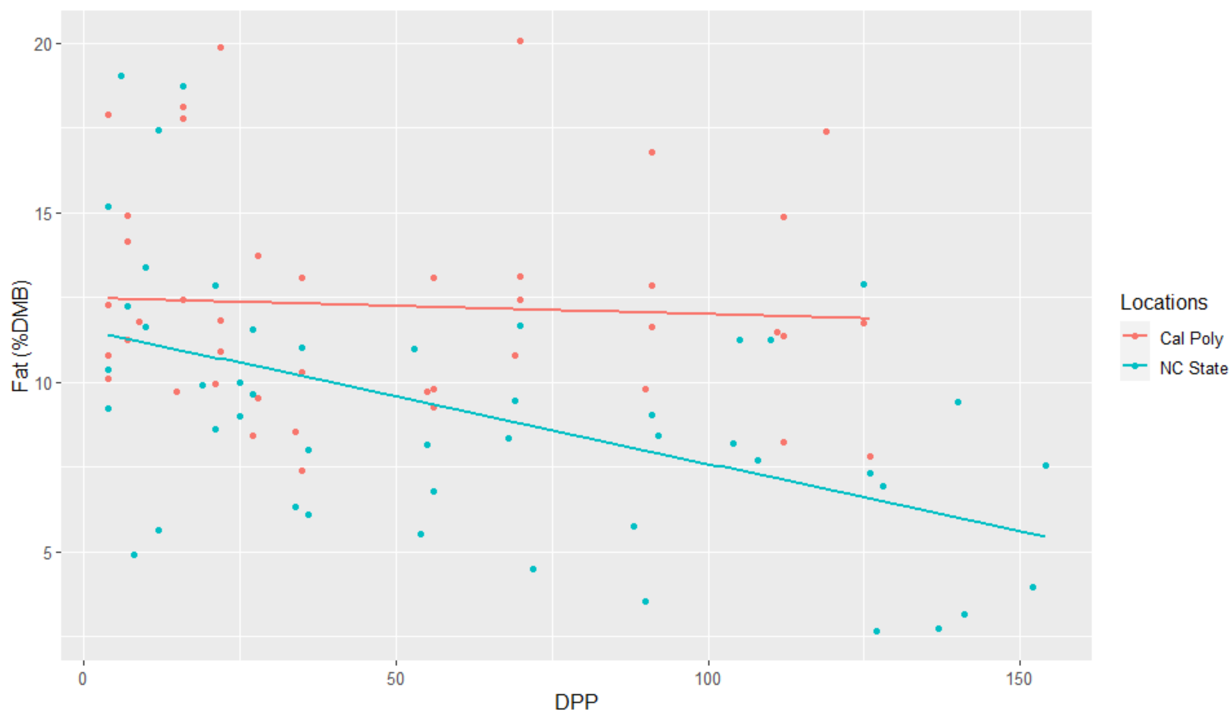


Figure 2.8. Percent fat concentrations (dry matter basis) of California Polytechnic State University (Cal Poly) and North Carolina State University (NC State) mares' milk over day post parturition (DPP) with associated regression lines (CA = 4, NC = 4) (n = 90 milk samples, ANCOVA, p < 0.05).

The range for sugar concentrations was between 29.58% DMB and 91.20% DMB, with the average = 59.69% DMB (6.59% milk). Sugar concentrations were relatively constant over time on a DMB (linear regression, R2 = 0.075, Pearson coefficient = 0.29). There was no difference in milk sugar concentrations (DMB) based on location (ANCOVA, $p = 0.35$) (Figure 2.11). There were 5 differences in sugar concentrations (DMB) among individual comparisons; between CA1 < CA3 ($p < 0.001$), NC1 < NC2 ($p = 0.02$), CA1 < NC1 ($p = 0.02$), CA1 < CA2 ($p = 0.001$), CA1 < NC3 ($p < 0.001$) (Figure 2.10).

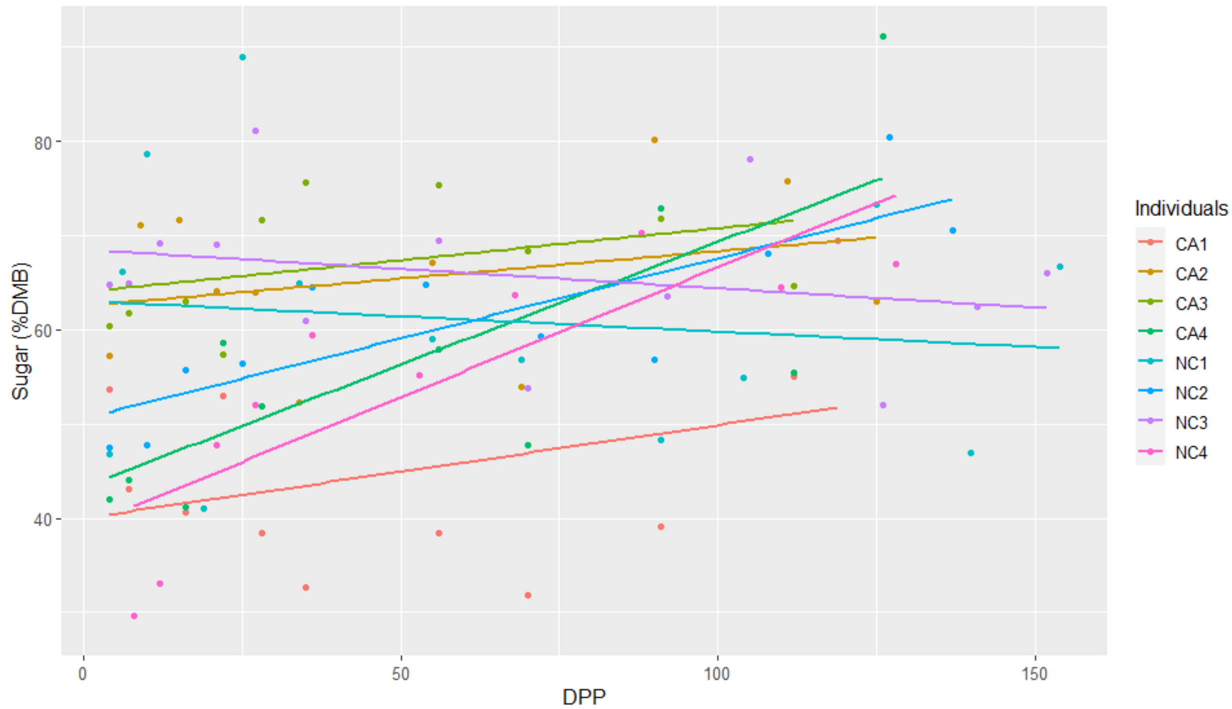


Figure 2.9. The trendlines and values of milk percent sugar concentrations (dry matter basis) among eight individual horses for up to 160 over day post parturition (DPP) at two housing locations (CA= 4 & NC= 4) (n = 90 milk samples, ANCOVA, p < 0.05).

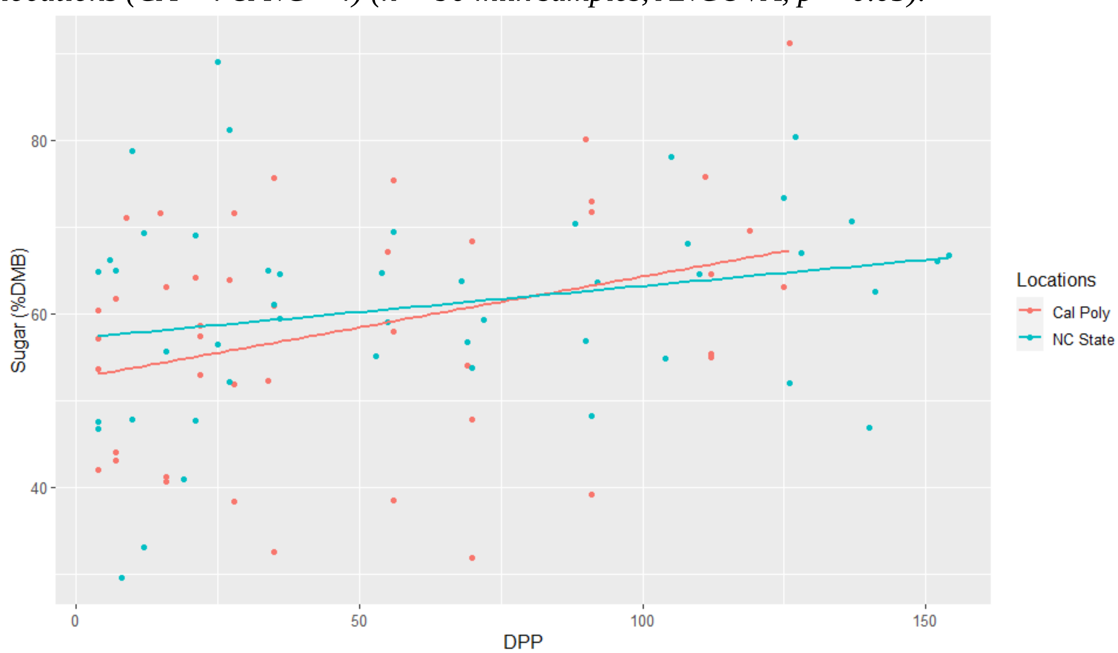


Figure 2.10. Percent sugar concentrations (dry matter basis) of California Polytechnic State University (Cal Poly) and North Carolina State University (NC State) mares' milk over day post parturition (DPP) with associated regression lines (CA = 4, NC = 4) (n = 90 milk samples, ANCOVA, p > 0.05).

The range for CP concentrations was between 9.98% - 54.97% DMB, with the average = 19.88% DMB (2.24% of total milk). Crude protein concentrations were relatively constant over time on a DMB (linear regression, R2 = 0.14, Pearson coefficient = -0.38). Cal Poly mares had significantly lower milk CP concentrations (DMB) than NC State mares (ANCOVA, p < 0.001) (Cal Poly = 15.68 % DMB, NC State = 20.48 %DMB) (Figure 2.13), however there were no differences in CP (DMB) among individuals (ANCOVA, p = 0.22) (Figure 2.12).

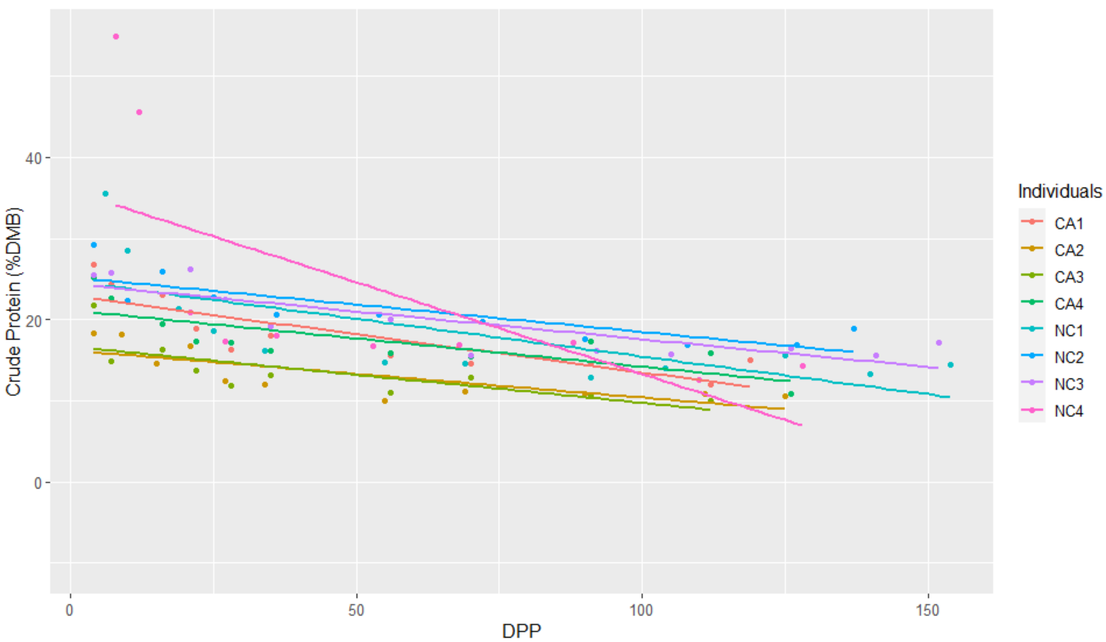


Figure 2.11. The trendlines and values of milk percent crude protein concentrations (dry matter basis) among eight individual horses for up to 160 over day post parturition (DPP) at two housing locations (CA= 4 & NC= 4) (n = 90 milk samples, ANCOVA, p > 0.05).

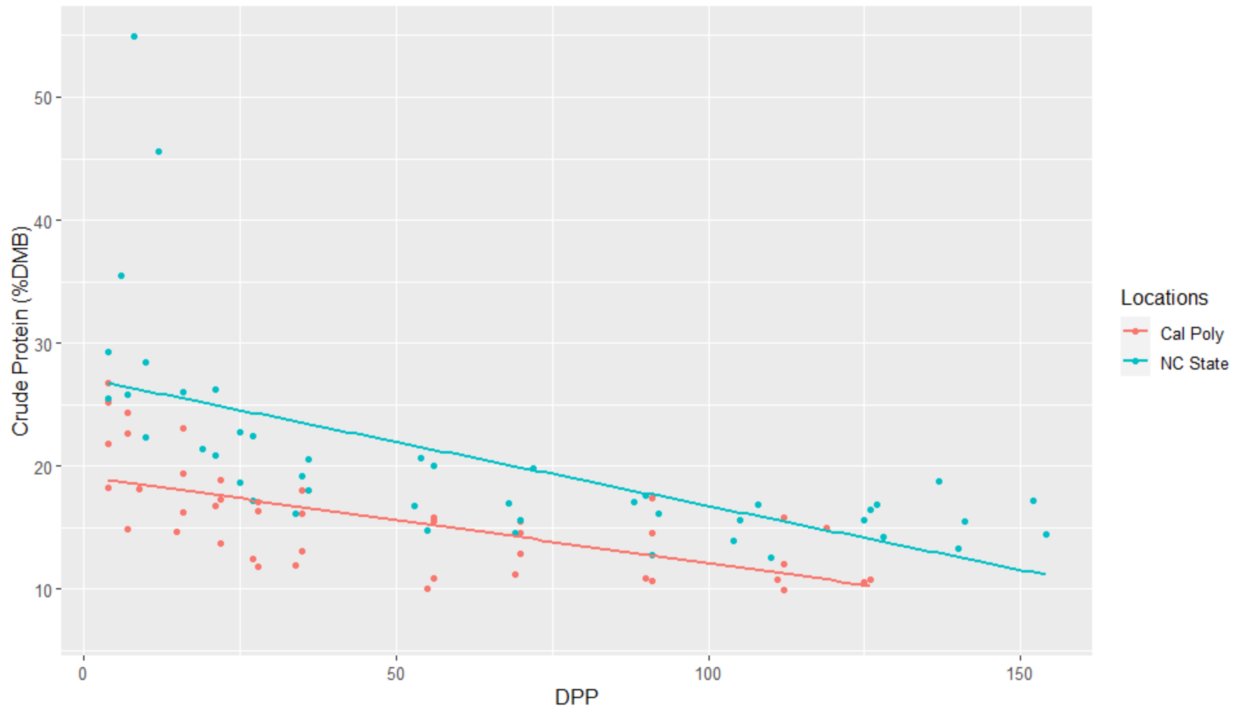


Figure 2.12. Percent crude protein concentrations (dry matter basis) of California Polytechnic State University (Cal Poly) and North Carolina State University (NC State) mares' milk over day post parturition (DPP) with associated regression lines (CA = 4, NC = 4) (n = 90 milk samples, ANCOVA, p < 0.05).

Table 2.2. The total median and range of milk macronutrients on a dry matter basis for all 8 mares representing two university equine farms (California Polytechnic State University vs North Carolina State University) for 3- 160 days post parturition.

Dry Matter		Sugar (% DM)		Protein (% DM)		Fat (%DM)	
Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
11.01	9.09 - 15.08	60.95	29.58 - 91.2	16.83	9.98 - 54.97	10.19	2.65 - 20.09

Ash (%DM)		Gross Energy (kcal/g)	
Median	**Range**	**Median**	**Range**
3.19	0 - 7.02	0.46	0.36 - 0.72

Table 2.3. The total averages (%) of milk macronutrients on a milk (fresh) and dry matter basis (DMB) for all 8 mares and at each institution over 160 days post parturition.

	Dry Matter	Sugar		Protein*		Fat*		Ash		Gross Energy
		Fresh	**DMB**	**Fresh**	**DMB**	**Fresh**	**DMB**	**Fresh**	**DMB**	**kcal/g**
Average	11.04	6.60	59.69	2.25	19.88	1.18	10.61	0.40	3.11	0.49
NC	11.19	6.76	60.90	2.34	20.48	1.00	9.10	0.42	3.60	0.52
CA	10.87	6.29	58.36	1.72	15.68	1.34	12.24	0.37	2.58	0.47

**There is a significant difference between NC and CA mares (ANCOVA<0.05).*

Discussion

Of the six macronutrients measured (DM%, Sugar, CP, Fat, Ash, GE), there was only a difference of California mares having higher overall fat concentrations and lower overall CP concentrations compared to mares housed in North Carolina. The cause of these differences may be due to a difference in diet composition. There could be enough variation in fat and CP concentrations that could explain the differences in milk macronutrient composition between locations. Both facilities provided a manufactured pelleted diet or supplement that were quite different in guaranteed analyses (Table 2.4). Purina Impact All Stages 12:6 Pellet (Purina, Arden Hills, MN) at NC State and Platinum Performance Equine (a supplement) (Platinum Performance, Buellton, CA) at Cal Poly. Both locations had similar quantities of alfalfa hay, and extensive pasture access. Cal Poly provided a proprietary grain mixture, where no equivalent additional feed was provided for NC State mares.Diets were offered, however a feed intake study was not conducted.

Table 2.4. Guaranteed nutritional analysis of Purina Impact All Stages 12:6 Pellet (Purina, Arden Hills, MN) and Platinum Performance Equine (Platinum Performance, Buellton, CA) manufactured diets for crude protein and crude fat composition.

	Platinum Performance	Purina Impact
Crude Protein	15.90%	12.00%
Crude Fat	30.00%	6.00%

The grain mix nutritional composition is unknown, however, given the pelleted diet and the serving sizes provided to the horses, we can hypothesize that there was a higher concentration of crude fat fed to the Cal Poly mares. The diet does not explain the increase of CP in the NC State mares’ milk.

A prior study successfully manipulated mare milk fat and protein concentrations through diet changes including supplemental fat and fiber diet and increased forage diet compared to concentrated pellets.[162] This may indicate that diet changes can be sufficient in altering mare milk macronutrient composition.

We compared the macronutrient composition among individual females to determine if there was major variance in milk macro nutritional composition based on individuals. There were slight variations in sugar, fat and DM concentrations, however; there were no individual trends or consistencies in the differences. In addition, the proportion of individuals that did have differences in macronutritional composition was lower than the proportion of individuals that did not have any differences (proportion different: DM = 3.6%, fat = 14.3%, sugar = 17.8%, CP = 0%, GE = 0%, ash = 0%). We conclude that although individuality can be a variable in milk macronutrient composition[159], it was is not a significant factor based on this study. This makes it promising that when making conclusions of overall species' milk compositions, individuality may be a minor factor.

Despite the differences of macronutrient composition among locations, the values were within the literature range (Table 2.5). [64,65,151,163]

Table 2.5. Mare milk macronutritional composition (%) summary from literature including microquantity assays compared to this study's average mare milk macronutritional composition results.

	Dry Matter	**Sugar**	**Protein**	**Fat**	**Ash**
Literature Range	10.50 - 24.90	6.37 - 7.00	1.85 - 2.31	1.06 - 1.70	0.32 - 0.42
Study Results	11.04	6.60	2.25	1.18	0.40

We can therefore conclude that although diet or location can alter the macro nutritional composition of mare's milk, it is negligible in altering the milk nutritional composition to be out of the normal range for the species. [64,65,151,163] This is an important conclusion for research intended to analyze limited numbers and quantities of milk samples from exotic species that may range in diets and locations.

Based on the results of this study we can conclude that microquantity measurement methods are an adequate way to assay milks from mares and possibly related species. Due to the nature of some of the ash, fat and CP assays, and their reduced dependency on machine calibrations, milk assays can be performed without traditional milk standardization protocols.[154] In addition, these assays were performed in duplicates, using less than 2 ml worth of milk. The general methods themselves also require little machinery or expensive equipment[154], making the ability to assess milk macronutrients accessible for laboratories or institutions that have limited machinery or resources. The microquantity measurements proved to be able to reliably execute mare milk macronutrient values that were within the estimated and normal range expected based on previous studies.[64] In addition, even with differences in and among diets and locations, the milk macronutritional composition was still in range of previous literature values. The values found in the study are also consistent with the milk composition of a healthy mare and foal since there were no major developmental or medical concerns with the test subjects. Colostrum was not included in the study since its macronutritional composition is much denser than milk that it would impact the data and provide misleading means.

The ability to analyze milk in low quantities is beneficial, especially for future analysis of endangered animal milks. In addition, the need for little – no machinery and accuracy make it more accessible to run assays in conservation institutions that may be more remote or have limited resources whether it is in other countries or on field sites. This study supports the methodology and the accuracy of milk micro assays with non – bovine milks.

Chapter 3: Milk Macronutrient Composition within the Perissodactyla Order

Abstract

Archived and donated zoological perissodactyla milk samples (n= 209) from 8 domestic horses (*Equus caballus*), 13 rhinoceroses (representing three species: *Diceros bicornis, Ceratotherium simum, and Rhinoceros unicornis*), 5 tapirs (representing two species: *Tapirus bairdii, Tapirus indicus*), and 16 wild equids (representing six species: *Equus ferus przewalskii*, *Equus grevyi*, *Equus quagga*, *Equus zebra*, *Equus africanus somaliensis*, *Equus asinus*) were collected from different institutions (n=16). These milk samples ranged from 3 to 200 days post parturition. Milk samples were analyzed at the Smithsonian National Zoo and Conservation Biology Institute (SZCBI) nutrition laboratory for major macronutrients: crude protein (CP), dry matter (DM), fat, sugar, and gross energy (GE). Analysis of variance (ANOVA) and Tukey post hoc tests were used to compare among both perissodactyla species and families (Equidae, Rhinocerotidae, Tapiridae). Overall, there was very little variation of milk macronutrient compositions within perissodactyla families, however there were significant differences when comparing between families. Large differences in the CP content between Tapiridae and Equidae milk were observed (Tapir: CP =5.93%, Fat=2.57%, Sugar=4.62%, Ash=0.68%, Domestic horse: CP = 2.25% Fat=1.22%, Sugar=6.35%, Ash=0.38%). These findings indicate that domestic equine milk is an insufficient substitute for tapirs. If equine milk were used as a replacer, modifications would be needed. This result is likely to have a high impact on emerging perissodactyla breeding and conservation programs with strong demand for milk formulas and replacements.

Introduction

Milk is vital to mammalian neonate survival as an evolutionary adaptation that provides the proteins, sugars, fat, vitamins, and minerals necessary for life.[18] Most institutions which manage breeding mammals have milk replacement protocols if a neonate needs access to supplemental milk.[5] Scenarios that require additional milk formula include: the neonate being orphaned, rejected, or sick, or if the dam is unable to produce adequate volume of milk or is sick herself.[4] In exotic animal husbandry, for many mammal species, care handbooks provide at least one suggestion for milk replacers and in conservation and breeding programs, these formulas are utilized to support the health and population of neonates from at-risk species.[7] However, there are significant differences in milk nutritional compositions among mammalian species and exotic mammal formulas are often dependent on a milk from a small subset of domesticated species whose macronutrient composition may not be appropriate.[2]

The taxonomic perissodactyla order has 13 of their 18 species listed as vulnerable, endangered, or critically endangered by the International Union for Conservation of Nature (IUCN) Red List.[8] Perissodactyla encompass the three families Equidae, Tapiridae, and Rhinocerotidae, all of which are odd – toed ungulates and hindgut fermenters.[42] The domesticated perissodactyla is the domestic horse, which has popularity as a companionship animal.[54] The milk composition of domestic horses has been thoroughly analyzed[64], however there is little milk research on other perissodactyla species.[12] Milk supplemental formulation recommendations intended for neonates of perissodactyla species including rhinos and tapirs are variable. [9,10,11] Their macronutrient composition often range between horse and bovine milks. [9,10,11] For example, rhino offspring from all species are often provided horse milk replacers, while tapir offspring from all species are sometimes given bovine milk replacers and equine milk replacer.[9] Without robustly comparing each species' true milk macronutrient composition, it is difficult to determine the best milk replacer match for any of the perissodactyla species. It is important to note that feeding incorrect milk formulas can result in malnutrition, inappropriate growth, and even death for neonates.[13] For conservation and breeding efforts to be effective, it is imperative to have an effective milk formula catered to each species if needed.

This research study analyzed the milk macronutrient composition of 12 of the 18 perissodactyla species and compared the composition specifically among the species and the three perissodactyla taxonomic families. This was conducted to determine if there are differences in milk macronutrient composition and, if so, which of these differences may need to be further examined in order to produce future perissodactyla neonate milk replacers for successful conservation programs.

Materials and Methods

Test Subjects

Perissodactyla milk samples were retrieved from the Smithsonian Milk Repository archive at the Smithsonian Zoo and Conservation Biology Institute (SZCBI, Washington, DC). In addition, milk samples were recruited from managed facilities across the United States with perissodactyla breeding programs or reported to have archived perissodactyla milk samples (Table 3.1). All institutions were provided milking collection protocols and acquisition forms to track neonate date of birth, sample date, location, and any other notes on the female or calf health. Samples were included in this research project if the milk was collected between 3 to 200 days post parturition (DPP) to exclude colostrum samples. This is the largest milk sample size of perissodactyla species to be analyzed.

Table 3.1. Perissodactyla milk sample metadata including the number of females, managed institutions, and total milk samples (n) analyzed. The collection date ranges and ranges of total days post parturition (dpp) are also reported.

Family	Species (Common / Scientific Name)	Females	Institutions*	Year	dpp	n
Equidae	**7**	**24**	**8**	**1995 - 2022**	**4 - 176**	**113**
	Miniature Ass *(Equus asinus)*	2	1	2003	18 - 56	2
	Somali Wild Ass *(Equus africanus)*	5	1	2022	129 - 176	5
	Domestic Horse *(Equus caballus)*	8	2	2022	4 - 154	90
	Przewalski Horse *(Equus ferus przewalskii)*	1	1	2018	4--87	8
	Grevy's Zebra *(Equus grevyi)*	6	2	2022	54 - 91	6
	Mountain Zebra *(Equus zebra)*	1	1	1996	16	1
	Plains Zebra *(Equus quagga)*	1	1	1995	31	1
Rhinocerotidae	**3**	**13**	**11**	**1985 - 2022**	**3 - 199**	**73**
	Black Rhino *(Diceros bicornis)*	6	6	1985 - 2020	3-163	20
	Greater One Horned Rhino *(Rhinoceros unicornis)*	3	3	2012 - 2022	2 - 199	20
	White Rhino *(Ceratotherium simum)*	5	4	2008 - 2022	35 - 195	33
Tapiridae	**2**	**5**	**5**	**1981 - 2020**	**3 - 103**	**23**
	Baird's Tapir *(Tapirus bairdii)*	3	3	1992 - 2020	3 - 103	18
	Malayan Tapir *(Tapirus indicus)*	2	2	1981 - 1999	6 - 101	5

**Samples were donated from 16 institutions: Busch Gardens (Tampa, FL), California Polytechnical Equine Center (San Luis Obispo, CA), Denver Zoo (Denver, CO), Detroit Zoo (Royal Oak, MI), Disney's Animal Kingdom (Lake Buena Vista, FL), Fort Worth Zoo (Fort Worth, TX), Franklin Park Zoo (Boston, MA), Hogle Zoo (Salt Lake City, UT), Miami Metro Zoo (Miami, FL), Milwaukee Zoo (Milwaukee, WI), North Carolina State University Equine Unit (Raleigh, NC), Potter Park Zoo (Lansing, MI), Sedgwick County Zoo (Wichita, KS), Smithsonian National Zoo and Conservation Biology Institute (Washington DC), St. Louis Zoo (St. Louis, MO), White Oak Conservation (Yulee, FL)*

Milk Collection

Facilities were provided milk collection protocols and acquisition forms standardized by the Smithsonian Nutrition Laboratory.[150] Milk collection directions were to clean the nipple area with clean water and then dry prior to collection. Manual expression was done by applying pressure to the area surrounding the nipple and squeezing firmly towards the nipple. Oxytocin was used for some samples. It was encouraged to extract milk from one gland, however, many samples were pooled by collecting in the same container. The milk was collected into clean, tight sealed sample tubes or bottles and frozen immediately at a minimum of -5°C.

The milk was archived and frozen at – 28.8°C at the SZCBI Milk Repository. The number of thaws for each historically archived sample is unknown, but they were thawed to room temperature a minimum of 3 times. Whenever transported, the milk was kept on dry ice or ice packs in insulated packaging to keep milk frozen.

Nutritional Analysis

Milk DM, fat, CP, total sugar, GE, and ash were analyzed at the Smithsonian National Zoo and Conservation Biology Institute Nutrition Laboratory following the lab's standard protocols.[154] Crude protein was calculated by drying 20 µl milk in a forced-air drying oven at 100°C and running samples through an elemental analyzer (Model 2400; Perkin Elmer, Waltham, MA) to measure nitrogen (Dumas method).[154] Crude protein was determined by multiplying total nitrogen by the conversion factor 6.38.[164] Fat content was measured with a microfat Rose-Gottlieb procedure that was modified at the laboratory and has been validated.[154] Total sugar was measured through a phenol-sulphuric acid colorimetric procedure[155,160] using lactose monohydrate standards and read at 490 nm on a microplate reader (Model ELX808; BioTek, Winooski, VT). Ash was determined by placing dried milk in a muffle furnace at 550°C for 8 hours. Dry matter was both measured and calculated throughout sample analysis when drying the milks while subsampling for CP analysis and ash. The GE (kcal/g milk) was calculated using the formula: GE = (9.11 kcal/g * % fat + 5.86 kcal/g * % CP + 3.95 kcal/g * % sugar)/100. To convert to kJ/g, multiply 4.18 kJ/kcal.[162]

Statistical Analysis

Using the statistical software R (The R Foundation, RStudio, Boston, MA), species and families had their mean, median, and range of macronutrient concentrations assessed for GE, DM, CP, sugar, fat, and ash. The general concentrations of macronutrients were assessed on a milk basis, dry matter basis (DMB), and gross energy basis (GEB).

Multilinear regression models were created to analyze the change of macronutrient composition over parturition time (dpp) within a family. Pearson correlations were calculated to assess the relationship between macronutrient composition trends over time (dpp) and how the macronutrients values trends correlate over time.

An analysis of variance (ANOVA) was conducted to compare among species and among families for each macronutrient. Subsequently, a post hoc Tukey's Honest Significant Difference test was conducted based on the proceeding ANOVA. Significant values were considered if the p value < 0.05.

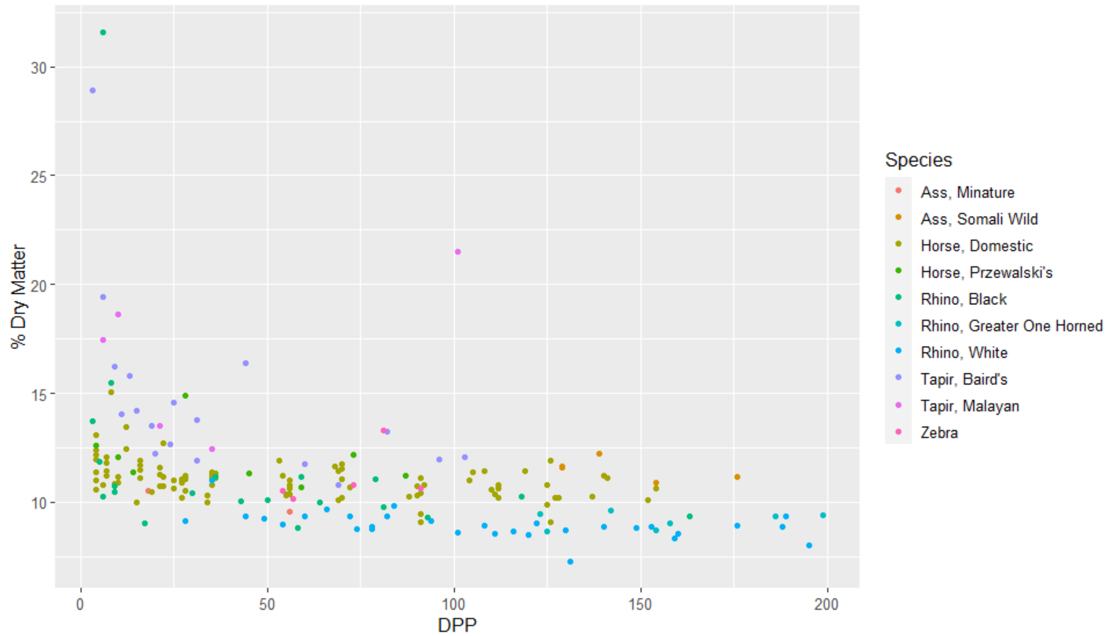
Results

The oldest perissodactyla milk utilized was from 1981, however, the median collection year of samples used (based on female parturition dates) was the year 2021, with the average being 2011. A total of 209 milk samples were analyzed and fit the criteria for being taken within 3 – 200 days post parturition to avoid colostrum sample bias during 0 – 3 dpp. 113 samples were in the Equidae family, 73 were from the Rhinocerotidae family, and 23 were from the Tapiridae family. A total of 42 females were sampled from 16 unique institutions. Equids had the most individual females (n=24), but the Rhinos had the most institutions (n=11). The tapir family had the least amount of diversity among females (n=5), institutions (n = 5), and samples (n = 23) (Table 3.1). There was a single milk sample from a mountain zebra (dpp = 16) and a single sample from a plains zebra (dpp = 31). For statistical analyses, we grouped all zebra species together (n = 8, dpp=16–91). For this research study, samples from the following perissodactyla species were unable to be collected: : Onager (*Equus hemionus*), Kiang (Equus kiang), Sumatran rhino (*Dicerorhinus sumatrensis*), Javan rhino (*Rhinoceros sondaicus*), lowland tapir (*Tapirus terrestris*), and mountain tapir (*Tapirus pinchaque*). We were able to collect from domestic horses (*Equus caballus*), black rhino (*Diceros bicornis),* white rhino *(Ceratotherium simum)*, greater one horned (*Rhinoceros unicornis*), Baird's tapir (*Tapirus bairdii),* Malayan tapir *(Tapirus indicus*), Przewalski horse (*ferus przewalskii),* Grevy zebra *(Equus grevyi), Equus quagga, Equus zebra,* Somali wild ass *(Equus africanus somaliensis),* and miniature donkey *(Equus asinus*).

The following significant differences among species when analyzing total means between 3 – 200 dpp for DM, sugar, fat, CP, ash, and GE (ANOVA, $p < 0.001$) were noted.

For milk GE concentrations (Table 3.2), there was a significant higher GE value in post – hoc Tukey test among Baird's tapir compared to: the Somali wild ass ($p = 0.001$), domestic horse ($p < 0.001$), greater one horned rhino ($p = 0.001$), white rhino ($p = 0.001$), and zebra ($p = 0.001$). There was also a difference with the white rhino having less GE than the: domestic horse ($p = 0.01$), P horse ($p =0.04$), black rhino ($p = 0.001$), and Malayan tapir ($p =0.01$).

For DM, there was a significant difference among all three families (ANOVA, $p < 0.01$). Tapirs had the highest DM on average (13.77) and rhinos had the lowest (9.31) (Table 3.2). Within the rhino family there was a difference in DM between the white rhino and black rhino (ANOVA, $p = 0.005$), black rhinos had higher DM content (white rhino = 8.96%, black rhino = 11.73%). There was one difference among the equid family species in DM with the Przewalski horse ($p = 0.03$) having higher DM than the domestic horse.



**Zebra = 3 zebra species (Plains, Grevy's, Mountain) are pooled.*

Figure 3.1. The percentage of milk dry matter over days post parturition (dpp) of 10 perissodactyla species.

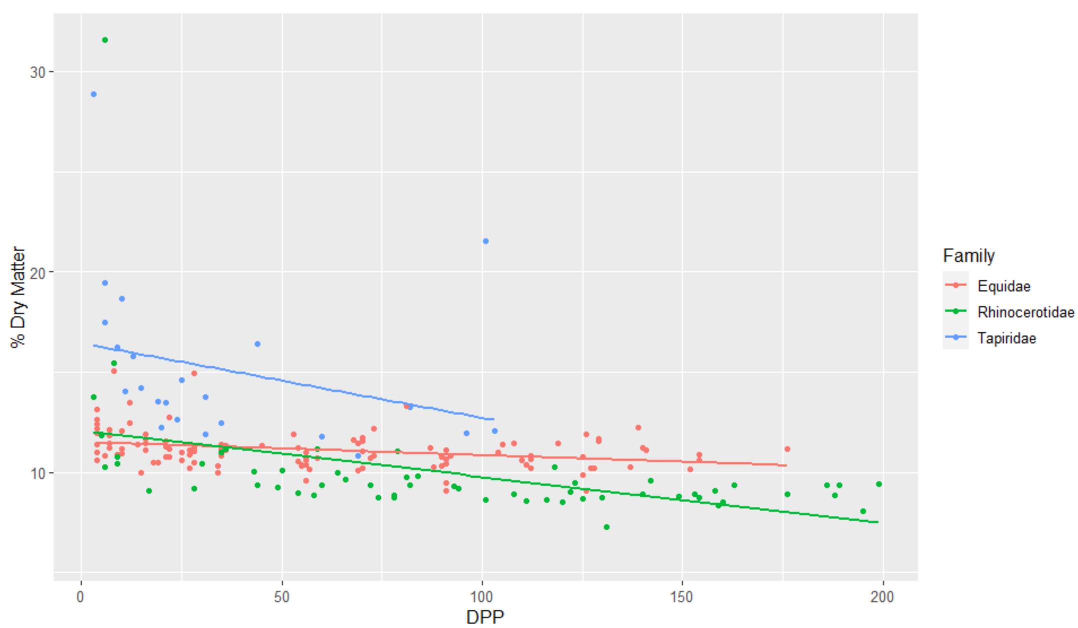


Figure 3.2. The percent dry matter content over time (days of parturition (DPP)) among perissodactyla families Equidae, Rhinocerotidae, and Tapiridae with linear trendlines (ANOVA, $p < 0.05$).

For milk sugar concentrations (sugar on a DMB) (Table 3.2), there was a significant Tukey post-hoc difference among Baird's tapir and the following species: domestic horse ($p < 0.001$), black rhino ($p < 0.001$), greater one horned rhino ($p < 0.001$), and white rhino ($p < 0.001$). The sugar concentrations ranged from 5.7% to 91.2% of milk dry matter with the white rhino having the highest sugar mean (74.4%), while the Baird's tapir had the lowest sugar mean (33.2%). The Tapiridae family had significantly lower sugar concentrations than the Equidae and Rhinocerotidae family. There was no difference in sugar concentrations between Equidae and Rhinocerotidae families.

For crude protein milk concentrations (CP on a DMB) (Table 3.2), there was a significant Tukey post -hoc difference among Baird's tapir and the following: domestic horse ($p = 0.001$), black rhino ($p = 0.001$), greater one horned rhino ($p < 0.001$), white rhino ($p = 0.001$), Przewalski horse ($p = 0.001$), miniature ass ($p = 0.04$), zebra ($p = 0.001$), and Somali wild ass ($p = 0.001$). There was also a Tukey post-hoc difference between Malayan tapir and zebra ($p = 0.02$), greater one horned rhino ($p = 0.004$), white rhino ($p = 0.001$), and Somali wild ass ($p = 0.02$). There was a significant Tukey post-hoc difference between the black and white rhino, with the black rhino having higher protein ($p = 0.02$). In addition, domestic horse milk has more CP than white rhinos ($p = 0.004$). There was a significant difference among all of the families, with Equidae having the lowest CP, followed by Rhinocerotidae, and Tapiridae having the highest.

For milk fat concentrations (fat on a DMB) (Table 3.2), with an ANOVA Tukey post - hoc test, Baird's tapir had greater concentrations than white rhino and greater one horned rhino ($p = 0.001$). According to ANOVA Tukey post hoc, the Przewalski horse milk had significantly more fat than the white rhino ($p = 0.01$), and greater one horned rhino ($p = 0.03$). There was also less fat in the white rhino milk than the black rhino based on an ANOVA Tukey post-hoc test ($p = 0.01$).

For milk ash concentrations (ash on a DMB) (Table 3.2), There was a significant difference in the Tukey post hoc test among Baird's tapir and: miniature ass ($p = 0.006$), Somali wild ass ($p = 0.001$), domestic horse ($p = 0.001$), black rhino ($p = 0.001$), white rhino ($p=0.001$), greater one horned rhino ($p = 0.001$), zebra ($p = 0.001$), and P horse ($p = 0.001$). There was also a greater milk ash concentration in Malayan tapir and Somali wild ass ($p = 0.001$), black rhino (Tukey post-hoc, $p = 0.03$), white rhino ($p = 0.001$), and greater one horned rhino ($p = 0.001$). Domestic horses had higher ash than white rhinos ($p = 0.001$).

There was a negative correlation for DM with calf age in the tapir and rhino families (Figure 3.2). This was consistent with fat and CP decreasing as sugar increased for both the tapir family (Pearson coefficient = -0.65) and rhino family (Pearson coefficient = -0.76) over the duration of lactation on a DM basis (Figure 3.3 and 3.4). Rhino sugar concentrations were steadily increasing over time on a DM basis (Figure 3.3). Since tapir milk had a decreasing trend of CP with an increasing sugar concentration on a dry matter basis, the trendlines intercept at approximately 65 days post parturition, where tapir milk becomes a high sugar milk instead of a high protein (Figure 3.4). The trendlines for equid milk concentrations on a dry matter basis over time were relatively constant with little change in concentration proportions (Figure 3.5).

Table 3.2. The total averages of milk macronutrients (dry matter (DM), sugar, protein, fat, ash, gross energy (GE)) on a milk and dry matter basis for perissodactyla species over 200 days post parturition. (% = percentage) basis, % GE = percent of gross

Family	Species	Dry Matter	Sugar			Protein			Fat			Ash		GE
			%	% DM basis	% GE	%	% DM basis	% GE	%	% DM basis	% GE	%	% DM basis	kcal/g
Equidae		**11.11**	**6.35**	**57.65**	**53.14**	**2.14**	**18.91**	**24.42**	**1.22**	**10.79**	**22.45**	**0.38**	**2.89**	**0.49**
	Miniature Ass	10.05	5.51	55.09	56.25	2.37	23.41	35.62	0.35	3.46	8.14	0.14	0.73	0.39
	Somali Wild Ass	11.51	5.37	46.97	60.78	0.95	8.27	16.04	0.87	7.55	23.19	0.18	1.23	0.35
	Domestic Horse	11.04	6.60	59.69	53.28	2.25	19.88	25.06	1.18	10.61	21.66	0.40	3.11	0.50
	Przewalski Horse	12.05	5.94	49.56	40.22	2.40	19.92	23.94	2.19	19.44	35.84	0.39	2.82	0.59
	Zebra*	11.09	4.99	45.58	62.34	0.93	8.57	17.52	0.71	6.36	20.14	0.21	1.58	0.32
Rhinocerotidae		**9.94**	**6.40**	**67.84**	**64.00**	**1.75**	**17.08**	**22.66**	**0.90**	**7.02**	**13.36**	**0.28**	**2.50**	**0.44**
	Black Rhino	11.73	6.06	57.59	50.67	2.81	23.96	28.72	2.10	12.05	20.61	0.36	3.02	0.59
	Greater One Horned Rhino	9.19	7.05	76.87	71.29	1.47	16.01	22.02	0.46	3.14	6.70	0.24	2.62	0.39
	White Rhino	8.96	6.47	72.43	70.52	1.16	12.88	19.09	0.42	4.64	10.39	0.23	2.14	0.36
Tapiridae		**15.09**	**4.62**	**32.83**	**28.02**	**5.93**	**38.31**	**43.77**	**2.57**	**15.98**	**28.20**	**0.68**	**4.02**	**0.75**
	Baird's Tapir	14.64	4.46	33.19	27.09	6.32	41.06	44.61	2.49	16.45	28.30	0.69	4.46	0.77
	Malayan Tapir	16.72	5.17	31.55	31.22	4.55	28.41	40.92	2.91	14.32	27.86	0.65	2.43	0.68

**Zebra = 3 zebra species (Plains, Grevy's, Mountain) are pooled.*

Table 3.3. The total median and range of milk macronutrients (dry matter, sugar, protein, fat, ash, gross energy) on a dry matter basis for perissodactyla species averaged 3 – 200 days post parturition.

Family	Species	Dry Matter		Sugar (% DM)		Protein (% DM)		Fat (%DM)		Ash (%DM)		Gross Energy (kcal/g)	
		Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Equidae	**7**	**11.03**	**9.09 - 15.08**	**57.41**	**29.58 - 91.20**	**16.61**	**4.44 - 54.97**	**10.16**	**2.65 - 33.38**	**2.98**	**0 - 7.02**	**0.46**	**0.25 - 0.83**
	Miniature Ass	10.05	9.6 - 11.12	55.09	48.81 - 61.38	23.41	19.42 - 27.4	3.46	2.95 - 3.97	0.73	0 - 1.46	0.38	0.37 - 0.39
	Somali Wild Ass	11.57	10.9 - 12.23	50.21	30.66 - 54.22	7.6	7.25 - 11.15	6.95	6.42 - 10.22	1.64	0 - 2.06	0.35	0.31 - 0.36
	Domestic Horse	11.01	9.09 - 15.08	60.95	29.58 - 91.2	16.83	9.98 - 54.97	10.19	2.65 - 20.09	3.19	0 - 7.02	0.46	0.36 - 0.72
	Przewalski Horse	11.73	10.69 - 14.92	48.94	42.36 - 57.42	19.00	14.31 - 25.06	17.19	14.84 - 33.38	3.01	0 - 3.89	0.56	0.50 - 0.83
	Zebra*	10.66	10.16 - 13.29	40.81	37.55 - 66.04	9.25	4.44 - 12.13	5.91	3.89 - 10.64	1.67	0 - 2.84	0.31	0.25 - 0.37
Rhinocerotidae		**9.31**	**7.27 - 31.6**	**71.66**	**8.04 - 86.37**	**15.27**	**8.35 - 50.22**	**4.569**	**0.58 - 75.69**	**2.58**	**0 - 5.15**	**0.39**	**0.17 - 0.71**
	Black Rhino	10.36	8.84 - 31.6	62.52	8.04 - 75.91	20.57	8.35 - 50.21	7.44	0.79 - 75. 69	3.04	0 - 5.15	0.47	0.38 - 0.71
	Greater One Horned Rhino	9.36	8.68 - 9.61	76.24	72.69 - 81.65	16.34	13.65 - 17.66	3.21	1.15 - 4.27	2.56	2.21 - 2.98	0.39	0.36 -0.41
	White Rhino	8.90	7.27 - 10.99	73.71	21.93 - 86. 37	12.11	8.35 - 18.56	4.48	0.58 - 10.46	12.11	0 - 3.82	0.36	0.17 - 0.44
Tapiridae		**13.77**	**10.81 - 28.89**	**36.75**	**5.71 - 43.98**	**37.68**	**11.80 - 69.09**	**15.9**	**3.70 - 30.89**	**4.41**	**0 - 7.49**	**0.67**	**0.46 - 1.55**
	Baird's Tapir	13.64	10.81 - 28.89	37.01	5.707 - 43.84	38.06	31.88 - 69.09	16.11	3.70 - 30.89	5.01	0 - 7.49	0.7	0.46 - 1.55
	Malayan Tapir	17.46	12.46 - 21.52	25.65	23.25 - 43.98	35.04	11.80 - 36.04	12.67	9.04 - 27.32	3.33	0 - 4.41	0.61	0.52 - 0.95

**Zebra = 3 zebra species (Plains, Grevy's, Mountain) are pooled.*

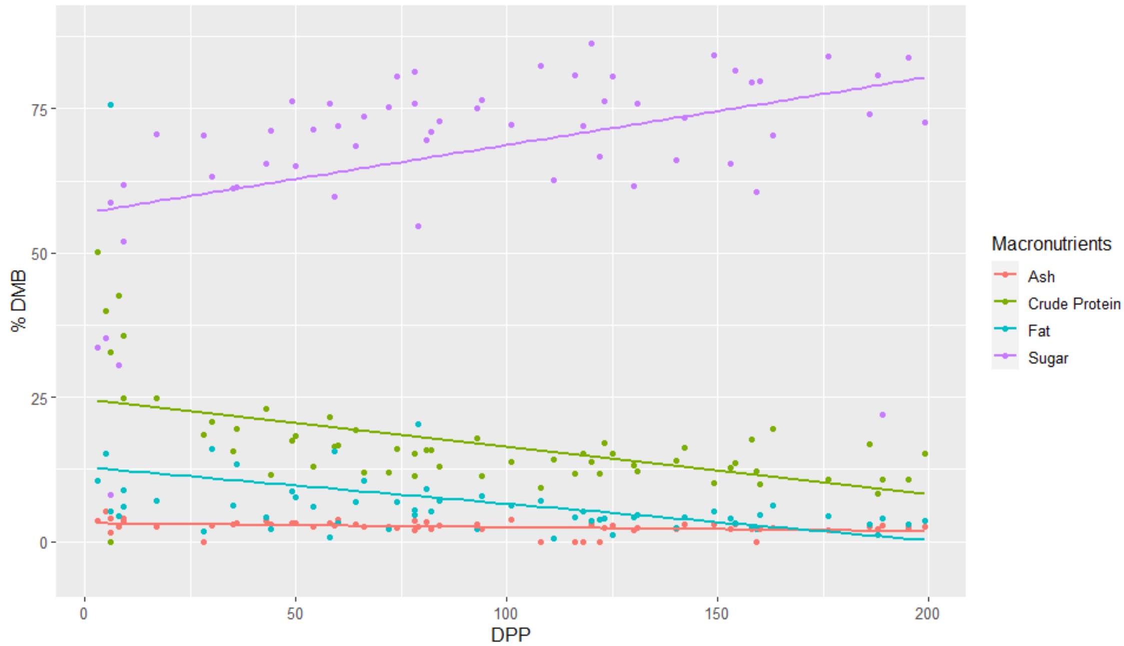


Figure 3.3. *Rhinocerotidae family macronutrient (ash, crude protein, fat, and sugar as percentage dry matter basis (% DMB) composition over days post parturition (DPP) (range=3–200).*

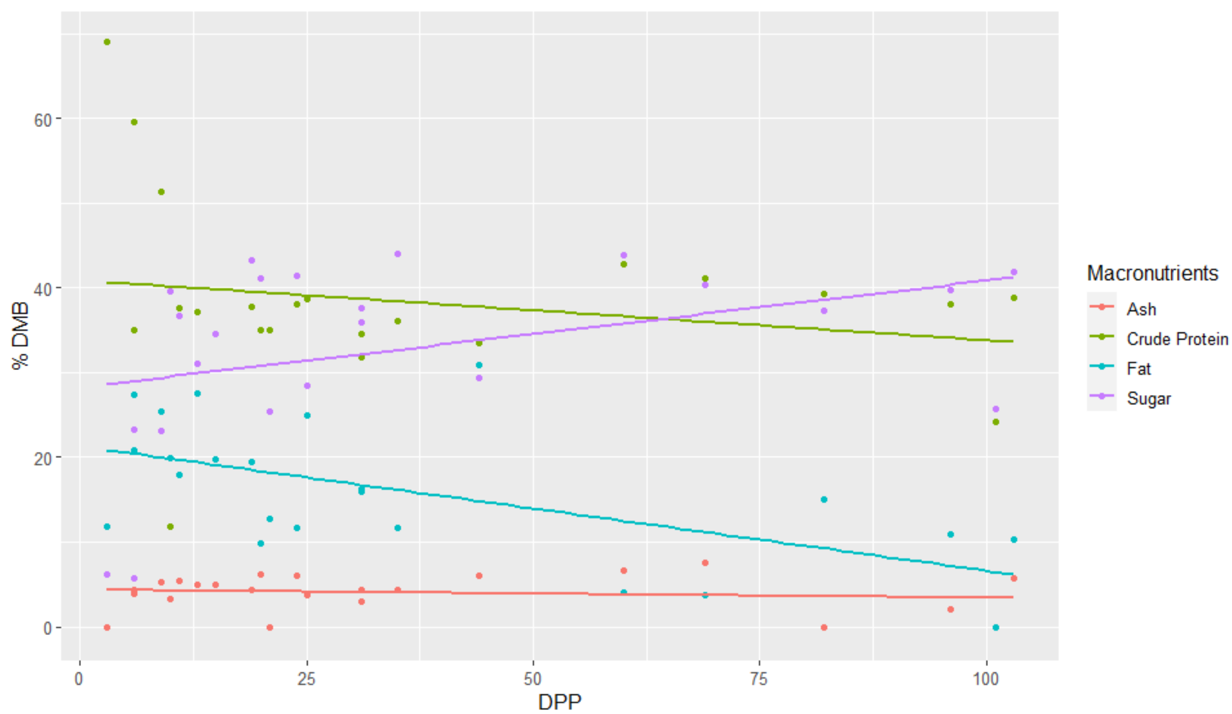


Figure 3.4. *Tapiridae family macronutrient (ash, crude protein, fat, and sugar as percentage dry matter basis (% DMB) composition over days post parturition (DPP) (range=3–200).*

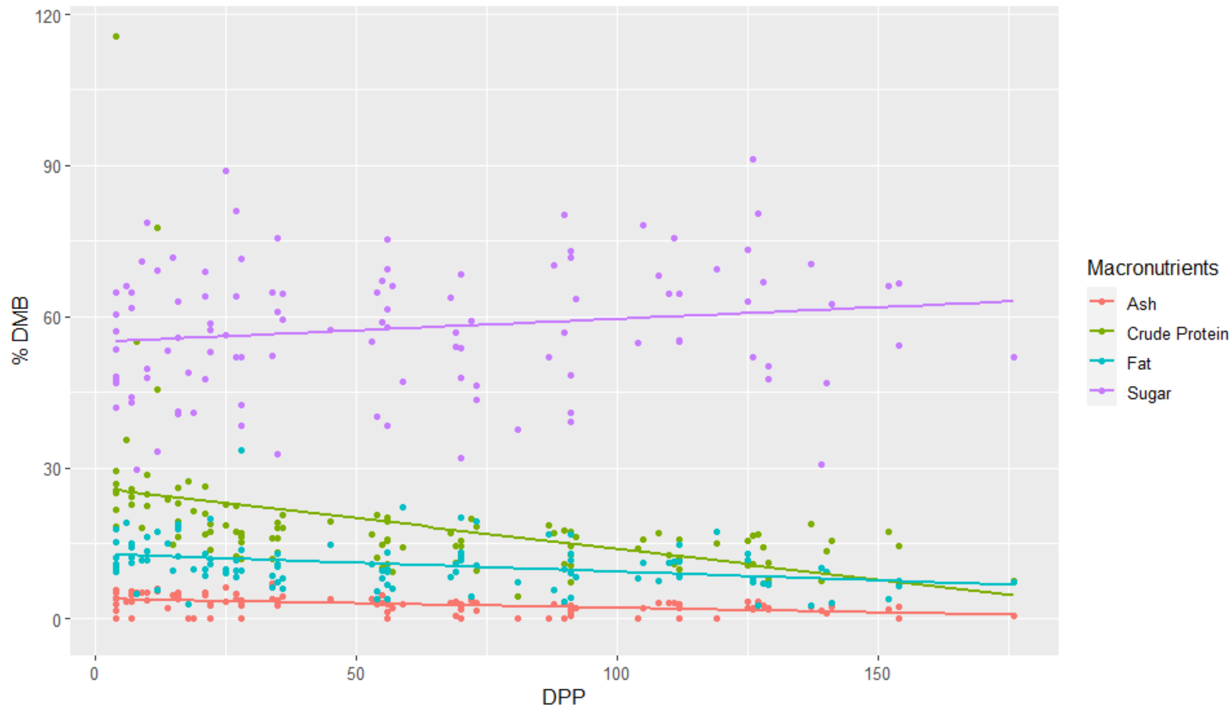


Figure 3.5. Equidae family macronutrient (ash, crude protein, fat, and sugar as percentage dry matter basis (% DMB) composition over days post parturition (DPP) (range=3–200).

Between the Equidae and Rhinocerotidae families there was a difference in CP, ash, and GE, however there was not a difference in sugar nor fat concentrations (Table 3.2). Rhinocerotidae had less CP compositions (mean: 35.5 < 55.6), less GE (mean: 0.44 < 0.49) and less ash concentrations (2.5 < 2.89) compared to Equidae (Table 3.2). Between Tapiridae and Equidae, there was a difference with Tapiridae having higher content in CP, fat, ash, and GE and lower content in sugar than Equidae. There was also a difference between the Tapiridae and Rhinocerotidae, with Tapiridae having higher concentrations in CP. Tapiridae milks had higher CP and lower sugar than the rest of the perissodactyla families.

In addition, when comparing the average nutrient concentrations on a GE basis (Table 3.2), both Equidae and Rhinocerotidae have over 50% of their energy accounted by sugar (sugar (%GE = 53.14%; 67.84%), protein is the second highest percentage averaging 24.42% and

22.66% respectively. Fat on a GE basis is lowest for Equidae and Rhinocerotidae at 22.45% and 13.36%, respectively. Tapiridae milk on a GE basis is more evenly distributed with 38.31% of GE being accounted for by protein, 32.83% by sugar, and 38.20% by fat, with the consideration that a complete even distribution would be a percentage of 33.

Discussion

The hypothesis that there are significant differences in milk macronutrient compositions among the perissodactyla families and species was supported. This implies that no single milk replacer will be suitable for all species in this order.

There were interesting trends in the changes of milk macronutrients over time. Within the Equidae family, the macronutrient change over time was minimal, the sugar concentration specifically was relatively constant (Figure 3.5). There was a slight decline in CP concentrations. Rhino milk increased in sugar concentrations over time and had a negative correlation with CP and fat (Pearson coefficient = 0.76; Figure 3.3).

The change of milk composition over time is important to consider when developing a milk replacer. For equids, there is little need for adjustments to milk formula to adapt to the change over time, except for water concentrations, which is often accounted for in milk replacer instructions.[63] However, with rhino milk there is a trend of increased DMB sugar concentrations, while the CP and fat concentrations decline. When developing a milk replacer for all rhino species, it may be best to increase sugar concentrations, while reducing the concentrations of CP and fat on trend with our results over the first 200 days. In tapir milk, there was a more dramatic shift where in the beginning 50 days after parturition, tapir milk was highest in CP concentrations, however, the sugar concentrations rose and became the highest nutrient by day 103, while fat and CP decreased. This suggests that a tapir milk replacer would need to shift from being a high CP to a higher sugar and lower fat milk at about 50 dpp.

Even with tapir milk developing into a high sugar milk over time, it still is a comparatively lower sugar concentrated milk ranging between 20 – 40% sugar on a DMB, whereas both rhino and equid milks range between 50 – 80% sugar.

When assessing the milk composition over time on a GE basis, the proportion of CP in tapir milk is relatively constant, whereas the proportion of sugar increases to be second highest macronutrient present in milk. The milk macronutrient composition trends over time in rhino and equid milks on a dry matter basis are essentially the same on a gross energy basis. With the exception that there is an increasing percentage trend of sugar on a gross energy basis in equid milks.

When analyzing at the species level, there were significant differences within the Rhinocerotidae family with the black rhino having more fat. The black rhino also had higher CP. The black rhinos had the highest GE milk of the Rhinocerotidae family. The nutritional differences in milk may be attached to the difference in rhino diets, with white rhinos and greater one horned rhino being fed a grazer diet[104], while black rhinos are fed a browser's diet, which is often higher in energy.[103] However, even with different dietary habits, the nutritional diet formulations for all the rhino and perissodactyla species currently in existence are based on the NRC requirements for horses.[9]

The tapir milk concentration coincides with previous literature, that the tapir milk is overall a higher CP, and lower sugar milk compared to the rest of the perissodactyla order.[138] Prior literature as a whole used similar macroquantifity analysis methods.However, there was also a difference in CP values between Rhinocerotidae and Equidae, making it a particular interest when comparing perissodactyla milks. When considering milk replacers for tapirs, it may be most accurate to provide a formula that has more crude protein for at least the early lactation period of 3 – 50 dpp. Equine milk replacer is therefore not a suitable alternative. Goat milk replacer, another tapir milk replacer is 4.5% fat, 3.6% protein, and 4.3% sugar[165] and also is not a close representation of tapir milk composition. However domestic sow milk is 5.9% protein, 5.4% fat, and 4.6% sugar[30] and therefore, for at least early lactation in tapirs, of common domestic milk formula options, sow milk may be the closest comparison to tapirs.

Both pigs and tapirs are hindgut fermenters and are categorized together in the American Associations of Zoos and Aquariums Taxonomic Advisory Group (TAG), instead of tapirs being with their taxonomic relatives such as rhinos or equids.[10] The similarity in swine and tapir milk concentrations are not necessarily surprising when considering their similar physiology. However, when considering the concentrations of the CP, it is important to investigate the whey to casein protein ratios in the milks and milk replacers. Both swine and tapir milks may be high in CP, but the amino acid and protein constituents need to be further examined. There was a study indicating that the whey to casein protein ratio in lowland tapirs is 1.6:1,[138] which is very similar to the ratio in domestic horses 1.7:1[64], whereas sow milk reportedly has a ratio of 0.6:1.[166] More investigations on the ratios of tapir whey to casein protein ratios, especially within the first 3 – 50 dpp is warranted. Protein is of particular interest since it is the prominent composition of the tapir milk for their early lactation.

Overall, although there are similarities in milk composition within perissodactyla order families, there are also important differences. Tapirs are more distinct with a higher protein milk, with protein accounting for about 75% more of the milk energy compared to the other families. Feeding tapir calves on equine milk replacers might comprise growth, based on these results.

Future studies can be conducted with micronutrients and other macronutrients such as calcium and phosphorus to better understand the mineral composition of the milks. There can also be investigations on the differences in gross energy of the milks to the perissodactyla species growth rates during nursing periods.

Although this is the largest study of perissodactyla milks, there is still a need to expand the sample population to reduce margins of errors found by differences in parity, diet, and environment. In addition, more species could be added to have a complete assessment of the entire perissodactyla order. The milks were part of a repository and underwent multiple freezing and refreezing that would not greatly affect macronutritional composition but should be considered for future studies if micronutritional composition is conducted.

Overall, based on the macronutrient composition of perissodactyla milks, it can be concluded that equine milk replacers would not be the best match for tapirs. However, equine milk replacers are adequate formulas for wild equids and rhino species given available milk replacer options.

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