The dynamic changes of African elephant milk composition over lactation

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Declaration

"I **Sibusiso Kobeni** declare that the Master's Degree research dissertation or interrelated, publishable manuscripts/published articles, or coursework

Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification **Food Science** at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education."

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Abstract

Taxonomically the Eutheria clade is split into mainly two groups: the Euarchontoglires, the well-known mammals, and the Atlantogenata, with the Elephantidae family. Like all mammals, the Atlantogenata produce milk with nutrients for growth and development of their neonate. However, the milk of elephants is hardly comparable with the milks of the Euarchontoglires, and it is unknown whether this is typical of Atlantogenata. Elephant milk has been studied for the past half-century, and unique properties are still being discovered. Some elephant milk nutrients are unique, and changes over lactation makes it impossible to define a typical elephant milk composition.

The current research has shown that African elephant lactation may be divided into three stages: the colostrums of two or three day's post-partum, a twelve-month period of constant milk composition change, and mature milk thereafter until the end of lactation. The specific changes in milk composition of the individual elements of African elephant milk over lactation were observed to follow a particular trend. The milk density was almost constant over lactation. The milk ash and content of the major minerals, Na, K, Mg, P and Ca, increased over lactation. Vitamins were present in low concentrations, and increases over lactation might be dependent on the milk fat content. Vitamin E occurred in quantifiable amounts, with traces of vitamins A, D_3 and K. The total protein content of African elephant milk increased with progressing lactation, with caseins as the predominant protein fraction.

The milk carbohydrates of African elephant consisted of high amounts of lactose, isoglobotriose and oligosaccharides. The total carbohydrates steadily decreased over

lactation, with the oligosaccharides becoming the major fraction, due to the decrease of lactose, which reached an equal level as isoglobotriose.

The milk fat of African elephant increased with advancing lactation. The total content of saturated fatty acids changed from 72 % in colostrums up to 96 % after 19 months of lactation. The fatty acids of 10 carbons in length and shorter, increased during lactation, while those of 14 carbons and longer decreased, while lauric acid (12:0) expressed little change. These changes occurred in two phases; drastic changes from day zero to 9 months, and slow changes thereafter.

The fatty acid composition of the phospholipids fluctuated throughout lactation. The phospholipids of medium-chain fatty acids were present in low concentrations, compared to the triacylglycerides, while long-chain fatty acids were present in high concentrations. The sterols also showed a fluctuating trend, perhaps following the fluctuation of the phospholipid fatty acids. Because milk secretion of the elephant is stimulated by suckling, it is possible that these fluctuations might be linked to the restoration of the milk secretion cell membrane after secretion.

The energy levels of African elephant did not change much in the first ten months of lactation but increased thereafter due to the increase in protein, fat, and saccharides. Theoretical energy calculations were twice that of the experimental ones. The calculation formula, which was designed for milk with a nutrient content within the same order of magnitude as domesticated mammals and humans, seemed not suitable for the unique nutrient properties of the African elephant.

Contents

Chapter 1

LITERATURE REVIEW

1.1. Introduction

Milk is a complex fluid that contains all the nutrients needed for infant growth and development (Hinde *et al.,* 2013). These key nutrients include proteins, water, carbohydrates, salts, lipids and other miscellaneous constituents secreted by the mammary gland (Jenness, 1988). However, this complex fluid also contains antibodies, complex carbohydrates, and proteins such as lactoferrin and lactoperoxidase, which give milk it's protecting and preventing properties (Séverin & Wenshui, 2005). Milk from different mammalian species have been investigated and reports confirm that the composition varies in each species, due to unique nutritional and physiological requirements of the respective neonates (Lefèvre *et al.,* 2010).

Milk is of importance in the dairy industry, especially in the production of high-value milk related products. The majority of research in milk has focused mainly on the economically exploited dairy animals (Prado *et al.,* 2008). However, not all properties, including those of the aforementioned species are clearly understood. As a result, the study of milk of non-dairy mammals may help provide some answers. A good understanding of milk properties is imperative for fundamental research and for the development of dairy products that can meet the ever-increasing demand of the food industry (Bhat & Bhat, 2011). In addition, data from various species is

needed because it provides information that can be useful in designing infant formula which is required by hand-reared orphaned animals (Prosser *et al.,* 2008).

Elephants of the Elephantidae family are of the largest surviving placental mammals. Like most mammals, the elephant produces milk and it is hardly comparable with the milks of humans or any other non-dairy species (Osthoff *et al.,* 2005, 2007a). The milk composition of the African elephant has been found to contain distinct properties. McCullagh and Widdowson (1970) were the first to conduct a comprehensive study on African elephant (*Loxodonta africana*) milk (McCullagh & Widdowson, 1970). In this study, milk samples were collected from thirty culled African elephant cows with lactation stages that spanned between 2 and 36 months. The average milk composition of the African elephant reported by McCullagh and Widdowson (1970) was 5.1 % protein, 3.6 % lactose, and 9.3 % fat. According to this report, elephant milk has a thin watery fluid with a mild distinctive smell and a slightly bitter taste. The milk never forms a cream on standing but processes such as thawing and freezing cause some separation and normally clings to glassware.

It had been established that elephant milk continually changes to a great extent during lactation, making it nearly impossible to define a typical elephant milk composition. Changes were observed in all the nutritional parameters of the milk (McCullagh & Widdowson, 1970; Osthoff *et al.,* 2005, 2007a). Osthoff and co-workers were the first to carry out a comprehensive study in African elephant milk drawn from living elephants. The studies also provided more information on the sugar and protein composition of African elephant milk (Osthoff *et al.,* 2005, 2007a).

1.2. Mammalian milk evolution

Mammals are vertebrate animals that possess specialized milk-producing glands intended to feed the mammalian young (Fox & Mcsweeney, 1998). This group of mammals includes terrestrial and aquatic animals. Mammals first appeared 166 million years ago, but the mammalian evolution can be traced back to 310 million years from the synapsids era (Oftedal, 2002a; Lemay *et al.,* 2009). The Mammalia consist of two sub-groups or sub-classes, which include the Theria and Prototheria (Fox & Mcsweeney, 1998). The monotremes are representatives of Prototheria and include the duckbilled platypus and echidna species (Springer & Krajewsk, 2009). They have specialized reproduction strategies that facilitate egg laying and lactation, characterized by the synthesis of complex milk (Oftedal, 2002b). The milk composition of monotremes changes substantially over advancing lactation, to support the development of the young, at different stages of growth (Sharp *et al.,* 2014).

The Theria is divided into two infraclasses namely the Metatheria (marsupials) and Eutheria (placentals), both bearing live young (Lemay *et al.,* 2009). The split between the two lineages (Metatheria & Eutheria) occurred 140 million years ago (Lefèvre *et al.,* 2010). Marsupialian infants spend a short period in the uterus and are usually born in an altricial state, after which they move to a pouch where they attach to a teat (Nicholas *et al.,* 2012). This infraclass includes species such as kangaroos and opossums (Lemay *et al.,* 2009). Eutherian mammals have long gestation periods and have a deeply invasive placenta, which supports in utero development that results in the birth of a well-developed offspring (Wildman *et al.,* 2006). The different reproduction strategies used by these mammals have a direct impact on the composition of milk, as the immature young have different needs with regards to

development, adaptive immunity, and growth (Lemay *et al.,* 2009). The milk composition of placental species remains more or less unchanged over advancing lactation, apart from the colostrum. However, in Marsupialian milk, the protein and lipid content tend to increase throughout lactation while the carbohydrate content decreases (Messer & Nicholas, 1991).

1.3. Nutrient composition

1.3.1. Milk fat

1.3.1.1. Mammalian milk fat

Fat is a macronutrient, which consists of esters of glycerol and various fatty acids. Fat provides energy, bioactive lipids and fat-soluble nutrients for mammals. The amount and composition of fat in mammalian milk varies and variation usually depends on the stage of lactation, maternal diet, and species type. Lipids are emulsified in the aqueous phase as globules that contain triacylglycerols, retinol esters and cholesteryl esters (Jensen, 2002). These globules are secretory vesicles and are formed and secreted by mammary epithelial cells during lactation (Heid & Keenan, 2005). The Globules contain a triglyceride core and are coated by a thin membrane known as the milk fat globule membrane that is derived from the apical membrane of cells undergoing lactation. The 10-20 nm membrane protects the globules from enzymatic degradation and aggregation. The membrane composition consists of lipids and proteins from the plasma membrane and the cytoplasm (Yao *et al.,* 2016; Singh & Gallier, 2017). The diameter of the fat globule membrane in bovine milk ranges from 0.1 to 15 µm with an average diameter of 3-4 µm. The size distribution of fat globule differs with species type, lactation stage, and diet (Singh & Gallier, 2017). However, in African elephant milk, the

size of the fat globules was reported to be half that of the globules found in bovine milk (McCullagh & Widdowson, 1970).

Lipid globules are responsible for providing a system that is convenient for delivering large quantities of energy and other lipid-soluble constituents, such as vitamins (A, D, E & K) and carotenoids to the suckling mammalian young (Singh & Gallier, 2017). Small molecules like lactose apply a slight osmotic pressure and can be stored in large amounts in alveolar spaces following secretion (Heid & Keenan, 2005).

Triglycerides are the most abundant fat component in milk. The smooth endoplasmic reticulum is responsible for the synthesis of triglycerides which are stored in small droplets of lipids coated with proteins and polar lipids (Heid & Keenan, 2005). These droplets can combine to form cytoplasmic lipid droplets, and progressively become entangled with the plasma membrane. The crescent cytoplasm is a part of the cytoplasm in the milk globule fat that is created when the membrane closes behind the lumen when fat droplets are projected through the apical system (Zou *et al.,* 2012; Singh & Gallier, 2017).

1.3.1.2. African elephant milk fat

African elephant milk has a low-fat content and compared to other terrestrial mammalian species, it falls under the group with average fat content (Oftedal & Iverson, 1995). The fat content of the African elephant milk reported by Osthoff et al. (2005, 2007a) was 5.6 % at 4 days postpartum and 7.6 % at 47 days postpartum, which is lower than the average fat content reported by McCullagh and Widdowson (1970) of 9.3 %. At

12 months lactation, the fat content was 6.1 %, which falls within the average fat levels reported by McCullagh and Widdowson (1970), and the 17.1 % reported at 18 months lactation, which is much higher than any average observed by these authors (Osthoff *et al.,* 2007a). McCullagh and Widdowson (1970) also reported an increase in the milk fat of the African elephant milk, where the fat content was 6 % at 3 months of lactation and 14 % at 36 months of lactation. An increase in the fat content of the African elephant milk with advancing lactation appears to be a distinctive trait of the elephant, as a change from 7 % to 17 % was reported for the Asian elephant (Abbondanza *et al.,* 2012). In bovine milk, similar trends are observed where the fat content increased over advancing lactation, but a decrease was reported in the milk of pigs (Csapó *et al.,* 1996).

1.3.1.3. Milk fatty acid composition

The majority of fatty acids in milk are esterified to glycerol to form triacylglycerides. Moreover, the triglycerides account for more than 98 % of the total milk fat (Singh & Gallier, 2017). A variety of fatty acids have been explored in mammalian milk lipids with chain lengths ranging from 4 to more than 24 carbon units and from saturated to unsaturated fatty acids. However, the majority of some fatty acids occur in small amounts (German & Dillard, 2006). In addition, the concentrations of major fatty acids in milk fat differs between species (Fox *et al.,* 2015a).

More than half of the fatty acids in the milk of most mammals originate from the diet. In contrast, the complex and structured fats are relatively constant in the milk (Yao *et al.,* 2016). Acetyl-coenzyme A (acetyl-CoA) is the major precursor of fatty acid synthesis in all mammalian species. In ruminant species the acetyl-CoA is derived from acetate or the oxidation of β-hydroxybutyrate produced by microorganisms in the rumen, while in

non-ruminants the acetyl-CoA is derived from blood glucose *(*McManaman, 2009*;* Fox *et al.,* 2015a).

The milk fat of ruminants is characterized by high concentrations of short-chain fatty acids which are derived from microbial fermentation in the rumen (Wu *et al.,* 2016). However, the non-ruminant fatty acid profile contains no short-chain fatty acids (Fox *et al.,* 2015a). Monogastric milk lipids contain high levels of polyunsaturated fatty acids compared to ruminants, due to the high proportion of fatty acids that are derived from dietary fats via blood (Månsson, 2008). In addition, the milk fat of marine animals contains high concentrations of long-chain unsaturated fatty acids. This is to allow the lipids to remain liquid in milk at cold temperatures of their setting (Fox *et al.,* 2015a).

The African elephant milk fatty acid profile is different from most mammals whose fatty acid profiles have been published (Osthoff *et al.,* 2005). A high content of capric and lauric acids, as high as 80 % combined, may occur in elephant milk fat. The fatty acid composition of rabbit milk contains capric acid, but only half the amount compared to African elephant milk (McCullagh & Widdowson, 1970; Demarne *et al.,* 1978). Both the Rhinoceros *Ceratotherium simum* and *Rhinoceros unicornis* milk fat also contain high levels of capric and lauric acid (Klös *et al., 1*974). On the other hand, cow milk contains low amounts of medium-chain fatty acids compared to African elephant milk (Auldist *et al.,* 1998; Osthoff *et al.,* 2005). Changes in the fatty acid composition of milk fat may occur in most mammals, but none so extensive as in African elephant milk (Csapó *et al.,* 1995; Gorban & Izzeldin, 2001; Osthoff *et al.,* 2005, 2007a; Yuhas *et al.,* 2006; Varricchio *et al.,* 2007).

1.3.1.4. African Elephant Milk Fatty Acid Composition

Based on the findings by McCullagh and Widdowson (1970), the composition of the glycerides found in elephant milk contain 60-70 % capric acid, 13-22 % lauric acid and low quantities of fatty acids with longer chains. Osthoff et al. (2005, 2007a) later confirmed that the African elephant fatty acid profile consists of high concentrations of medium-chain fatty acids and low levels of long-chain fatty acids. In addition, both capric and lauric acid make approximately 60 % of the total fatty acid composition of the African elephant milk fat. Other fatty acids such as myristic, palmitic and oleic acids are found in small amounts. Capric acid was reported to be about 35 % of the total fatty acids during early lactation (4 and 47 days after birth), which is significantly lower than the average concentration reported by McCullagh and Widdowson (1970) of 50 and 70 % at 3 and 36 months post-partum respectively (Osthoff *et al.,* 2005). In the latest reports by Osthoff et al. (2005, 2007a, 2012) it was shown that the capric acid content increased over advancing lactation from 43.2 to 52.1 and 61.3 % at 12, 14, and 18 months respectively. The amounts provided above are lower than the 65 % reported by McCullagh and Widdowson (1970) at a similar period of lactation (Osthoff *et al.,* 2007a). According to Osthoff (2012), the difference in the data is not due to the difference in geographical origin or diet. The difference might be in sub-species since the studies by Osthoff et al. (2005, 2007a) were done on Southern African elephants (*Loxodonta africana*), while in previous reports, studies were done in elephants from East Africa (*Loxodonta africana knochenhaueri*) (Smithers, 2005).

1.3.2. Milk proteins

1.3.2.1. Mammalian milk proteins

Milk consists of different types of proteins and the majority of these are synthesized in the mammary gland (Farrell *et al.,* 2004). Major milk proteins found in mammalian species can be categorized into different groups namely the whey (α-Lactalbumin and β-Lactoglobulin being the major components), milk fat globule membrane (MFGM), and caseins (αs_1-CN, αs_2-CN, β-CN, and κ-CN) (D'Alessandro *et al.,* 2010). In bovine milk, these proteins represent approximately 92 % of the overall proteins in milk and the rest is represented by immunoglobulins, bovine serum albumin and enzymes (Bequette *et al.,* 1998).

Milk proteins exist in both the aqueous and lipid phase of milk, either in a colloidal or soluble state. In bovine milk, the caseins account for up to 80 % and whey protein only 20 % of the total proteins in milk (Fox *et al.,* 2015b). However, the whey protein fraction consists of low molecular weight peptides which are known as miscellaneous minor proteins such as lactollin, lactoferrin, transferrin, ceruloplasmin, M-1 glycoprotein epidermal growth factor, glycolactin, glycoprotein-A and kinogen (Farrell *et al.,* 2004). Other proteins are present in low quantities in milk. These proteins are transported via transcellular or paracellular mechanisms into milk (Bequette *et al.,* 1998). Most of these proteins are blood-derived and involve the immune system. The serum albumin and immunoglobulins form part of the proteins that are taken up by diffusion or active transport inside the mammary cell (Shennan & Peaker, 2000).

1.3.2.2. Casein

Milk contains different types of casein proteins that do not exist as individual entities but as colloidal aggregates of casein proteins and calcium phosphate called the casein micelle (Walstra, 1999). One of the key functions of the casein micelles is to act as a

transporter of nutrients such as calcium, phosphate and essential amino acids for, optimum growth of the mammalian young (De Kruif & Holt, 2003). The secretion of caseins into the mammary gland is in response to lactogenic hormones (Lefèvre *et al.,* 2010). Individual caseins in a casein micelle are different from each other, with each having a unique peptide sequence and physicochemical properties. The posttranslational modification of the serine and threonine groups, and factors such as casein gene mutation, are distinct with each casein fraction (Swaisgood, 1993).

Caseins have a high proline content, and therefore have little secondary structure (Farrell *et al.,* 2004). A three-dimensional structure using X-ray crystallography of the caseins cannot be elucidated due to the inability to crystalize caseins. However other methods, such as molecular modelling, small-angle X-ray scattering (SASX) and small-angle neutron scattering (SANS) can be utilized to predict the casein secondary structure (Swaisgood, 1993; Qi, 2007). Caseins are not globular in nature because they lack strategically placed cysteine residues, which are responsible for stabilizing the globular structure of proteins via disulphide bonds (Swaisgood, 1993).

α_{s1}-Casein

The α_{s1}-casein forms part of the calcium-sensitive casein family and this is due to their distinguishing character of greater solubility in the presence of calcium (Holt & Sawyer, 1988). In bovine milk, the α_{s1}-casein is the most abundant casein fraction constituting approximately 40 % of total casein (Farrell *et al.,* 2004). Bovine α_{s1}-casein consists of 199 amino acids and the sequence lacks cysteine or cysteinyl residues. However, the human α_{s1}- casein does not lack cysteine or cysteinyl residues, and since there is an absence of α_{s2}-casein in the human casein fraction, the α_{s1}-casein can form disulphide-linked

heteromultimers with the κ-caseins that contain a single cysteinyl residue (Rasmussen *et al.,* 1999; Martin *et al.,* 2003).

This casein component has multiple phosphorylation sites in its sequence. In bovine milk, α_{s1}-casein exists in two phosphorylated forms containing 8 and 9 bound phosphate groups respectively (Eigel *et al.,* 1984; Ginger & Grigor, 1999). In neutral pH buffer, the α_{s1}-Casein has the highest net negative charge, but only in the presence of monovalent cations (Farrell *et al.,* 2004).

The α_{s1}-casein has 3 hydrophobic domains, these being residues 1-44, 90-113 and 132-199 that are well conserved between species (Martin *et al.,* 2003). Bovine α_{s1}-casein contains a highly acidic region between residues 38 and 78 that is responsible for binding calcium (Farrell *et al.,* 2004). Based on circular dichroism (CD) or Raman (FTIR) spectral analysis there is a presence of approximately 14 % α-helix, 40 % β-sheet and 24 % turn-like structures in the α_{s1}-casein (Byler *et al.,* 1988). In addition, the α_{s1}-casein contains highly conserved 15 amino acid residue signal peptide and the rest of the mature sequences differs among mammalian species (Ginger & Grigor, 1999).

α_{s2}-Casein

In bovine milk, the α_{s2}-casein makes up to 10 % of the casein fraction and this casein component is highly phosphorylated. Two major and one minor phosphoforms of the same protein have been observed, with smaller degrees of intermolecular disulphide linkages (Swaisgood, 1993). The seryl residues in this protein component incorporate 10 to 13 phosphate groups (Farrell *et al.,* 2004). These phosphate moieties are grouped in three regions in the sequence (7-31, 55-66 & 129-143) (Martin *et al.,* 2003). The

phosphoseryl and glutamic residues that form three clusters of anionic groups make the α_{s2}-casein the most hydrophilic casein (Swaisgood, 1993).

The α_{s2}-casein fraction has been identified in several non-dairy species namely the guinea pigs (casein A), rabbits (α_{s2a}- and α_{s2b}-casein), rats (γ-casein) and mice (γ- and ε-casein (Ginger & Grigor, 1999). Human milk seems to lack α_{s2}-like casein so the comparison of the amino acid sequence is limited to a few eutherian species (Martin *et al.,* 2003). In addition, ovine milk contains two non-allelic forms of the α_{s2}-casein, which is due to an internal deletion of nine amino acid residues (Boisnard *et al.,* 1991). Based on the structural organization the genes responsible for encoding both the α_{s2}- and β-casein are closely related to each other, more than the α_{s1}-casein gene (Ginger & Grigor, 1999). Bovine α_{s2}-casein contains 207 amino acid residues and it is one of the last caseins to be sequenced (Martin *et al.,* 2003). Only four genetic variants have been reported for this casein fraction thus far and these are termed variants A-D (Eigel *et al.,* 1984). Both circular dichroism (CD) or Raman (FTIR) spectral analysis indicate a presence of approximately 30-40 % α-helix, 20 % β-sheet and 20 % turn-like structures in α_{s2}-casein (Byler *et al.,* 1988).

β-Casein

β-Casein is one of the major protein components in the human casein family, a characteristic shared with the African elephant (Lönnerdal, 2003; Madende *et al.,* 2015). The molecule lacks cysteine residues but rich in proline residues (Greenberg *et al.,* 1984). Bovine β-casein contains 5 phosphate molecules in its phosphorylated form, whereas other species have several phosphoforms of β-casein that contain different numbers of phosphate groups attached to ser/thr residues. Human β-casein contains up

to five phosphorylated sites, equine has seven phosphorylation sites and ovine has six (Mamone *et al.,* 2003; Girardet *et al.,* 2006; Poth *et al.,* 2008). The location of the phosphorylated clusters of ser/thr residues in the β-casein sequence is close to the N-terminal region (Sato *et al.,* 1991). In solution, the amphipathic nature of the β-casein allows this casein fraction to form detergent like micelle structures/aggregates (Farrell *et al.,* 2004). Enzymatic hydrolysis of the β-Casein results in the formation of multiple fragments called the γ-casein, which are not present during the synthesis of milk. They contain residues 29-209, 106-209, and 108-209 (Swaisgood, 1993).

Bovine β-casein contains 207 amino acids residues and out of the four caseins, the β-casein component is the most hydrophobic (Martin *et al.,* 2003). The β-casein found in African elephant milk and other species such as humans, mouse, and pig, contains an extended C-terminal which consists of multiple charged amino acids, thus making it more hydrophilic than the β-casein sequence found in bovine milk. Comparison of African elephant β-caseins hydropathy plots showed that the African elephant β-caseins contained more hydrophilic stretches than bovine β-casein (Madende *et al.,* 2015). Therefore, such properties may conclude that this casein component has a characteristic interaction inside the casein micelle that exposes the hydrophilic parts to the surface.

Bovine β-casein has been reported to have nine genetic variants (Martin *et al.,* 2013). Based on circular dichroism (CD) and Raman (FTIR) spectral analysis, bovine β-casein has approximately 15 % α-helix, 30 % β-sheet and 29 % turn-like structures (Byler *et al.,* 1988). In addition, the β-casein contains a highly conserved 15 amino acids long signal peptide, similar to that which is observed for α-caseins (Ginger & Grigor, 1999).

κ-Casein

The κ-casein is a calcium insensitive casein fraction which is located on the surface of the casein micelle where it provides stability and prevents coagulation (Farrell *et al.,* 2004). Bovine κ-casein contains 169 amino acids and its one of the most studied casein components in milk (Martin *et al.,* 2003). This casein component contains the lowest phosphate molecules compared to other caseins. The sites of phosphorylation are limited to the C-terminal region and they exist as single sites instead of clusters (Ginger & Grigor, 1999). In addition, κ-casein is the only casein component that contains carbohydrate moieties, and post-translational modification takes place on one or more threonine sites (Farrell *et al.,* 2004).

The κ-casein is sensitive to aspartate protease, chymosin cleavage (Miyoshi *et al.,* 1976). Cleavage occurs between specific Phe-Met residues in ruminant κ-casein or in Phe-Ile or Phe-Leu in other mammalian species (Jollés *et al.,* 1968). Products of the cleavage yield two fragments namely, the macropeptide or glycomacropeptide (C-terminal) which is glycosylated and highly charged and the hydrophobic para-κ-Casein (N-terminal fragment) (Nakhasi *et al.,* 1984).

African elephant contains low amounts of κ-casein, a characteristic it shares with species such as the humans, horse, and rats (Martin *et al.,* 2003; Madende *et al.,* 2015). Multiple sequence alignment shows that there is a 50 % sequence identity between bovine and African elephant κ-caseins, with the majority of the substitutions being conservative. Based on the observations made from the hydropathy plots, African

elephant κ-casein behaves and function the same way as κ-caseins found in all other species (Madende *et al.,* 2015).

Two major genetic variants exist for κ-casein in bovine milk termed A and B. The difference between the two variants is due to amino acid substitutions at residues 136 and 148 (Farrell *et al.,* 2004). Circular dichroism (CD) and Raman (FTIR) spectral analysis indicated that the secondary structure of the κ-casein has 15 % α-helix, 30 % ß-sheets and 25 % turn-like structures (Byler *et al.,* 1988). In addition, κ-casein contains a 21-amino acid long signal peptide unlike the calcium-sensitive caseins (Ginger & Grigor, 1999).

1.3.2.3. Casein micelle

Caseins in milk exist as colloidal aggregates of individual casein components and calcium phosphate called casein micelles (Dalgleish & Corredig, 2012). The composition of individual casein components within the casein micelle varies between species (Holt, 2016). Casein micelles have several biological functions such as being transporters of high amounts of calcium and phosphate to prevent the mammary gland from being calcified and to ensure the safe secretion of potentially fibrillogenic casein proteins via the mammary gland (Holt *et al.,* 2013). In addition, casein micelles provide adequate nutrition to the neonate due to its ability to form gels via acidification and proteolysis.

Casein micelle structure has been the subject of extensive research over the past years and details on a molecular level still remain vague (Qi, 2007). Based on the physicochemical properties of micelles, different conflicting models have been proposed to try to depict the bovine casein micelle structure (McMahon & McManus, 1998). The

different models fall into three general categories: sub-unit models, internal structure models, and coat-core models. Table 1.1 shows the amount of individual casein components and size of the casein micelle in different mammalian species.

Table 1.1 The casein levels of individual casein components and casein micelle size ([a]Madende, 2017; [b]Potočnik *et al.,* 2011; [c]Qi, 2007).

Species	α_{s1}- casein %	α_{s2}- casein %	β- Casein %	κ-Casein %	Casein Micelle (nm)
Cow	38[c]	10[c]	40[c]	12[a]	182[b]
Sheep	50[b]	+	40[b]	10[b]	210[b]
African Elephant	-	-	89[b]	11[a]	N/A
Human	3[c]	-	70[c]	27[c]	64[b]
Horse	40-60[b]	Trace	40-50[b]	4-7[b]	255[b]

The casein micelle size also varies amongst species. Human milk has the smallest casein micelles (approximate diameter 64 nm) compared to other species in Table 1.1. The horse casein micelles seem to have larger casein micelles with diameters of about 255 nm (Potočnik *et al.,* 2011).

1.3.2.4. Whey proteins

Protein components that remain soluble after the isoelectric precipitation of bovine milk caseins at pH 4.6 are known as whey proteins. This fraction consists of five major

protein components namely, α-lactalbumin, β-lactoglobulin, serum albumin, and Immunoglobulins (Farrell *et al.,* 2004).

Alpha-lactalbumin forms part of the major whey proteins and has a specific physiological function. It forms an interaction with the β-1,4-galactosyltransferase to form the lactose synthase complex. This complex is responsible for the synthesis of lactose from UDP-galactose and glucose (Rajput *et al.,* 1996; Urashima *et al.,* 2012b). The absence of α-lactalbumin results in the formation of N-acetyllactosamine a primary monomer responsible for oligosaccharide formation, through the transfer of the galactosyl residue from UDP-galactose by the -1,4-galactosyltransferase 1 (β-1,4-GT1) enzyme to N-acetylglucosamine (Urashima *et al.,* 2001). Therefore, the amounts of the α-lactalbumin present in the mammary gland has a direct influence on the concentrations of lactose and oligosaccharides present in milk (Stacey *et al.,* 1995; Ramakrishnan & Qasba, 2001). The α-lactalbumin shares similarities with the c-type lysozyme, on a genetic and structural level (Mackenzie & Lascelles, 1968).

Alpha-lactalbumin is a 123 amino acid residue globular protein with a molecular mass of 14.128 kDa (Brew *et al.,* 1970). The α-lactalbumin binds calcium and other metals, which is imperative for disulphide bond formation and proper folding (Hiraoka *et al.,* 1980; Chrysina *et al.,* 2000). The crystal structures of α-lactalbumin of non-bovine species have been determined, these include the baboon, guinea pig, goat and human (Pike *et al.,* 1996).

African elephant α-lactalbumin sequence contains high amino acid homology with other species (Madende *et al.,* 2015). Moreover, the amino acid residues on the active site of

the African elephant lactose synthase seemed to be conserved and not different from that of other mammalian species (Ramakrishnan & Qasba, 2001; Madende *et al.,* 2015). The 3D structure of the African elephant lactalbumin also show no major differences when compared to other species. In addition, the computer modelling of the African elephant α-lactalbumin and lactose synthase is similar to other mammals, therefore the synthase activity is assumed to be the same (Madende *et al.,* 2015).

1.3.2.5. Elephant milk proteins

The protein levels of the African elephant milk fall within the same range as that of most mammalian species. The different stages of lactation have an immense influence on the content and composition of proteins in the milk. A change in the ratio of casein and whey proteins had been observed in the elephant milk at different lactation stages. Therefore, a shift in the ratio from equal amounts in the early stages of lactation to 2:1 after 18 months was reported (Osthoff *et al.,* 2005, 2007a, 2012).

It had been established that the casein fraction of the African elephant milk, lacks α-caseins (Madende *et al.,* 2015, 2018). Moreover, β-casein was observed to be the major casein component in African elephant milk. African elephant β-casein exists in multiple variants and up to five isoforms have been deduced or detected (Madende *et al.,* 2018). The κ-casein is present in low quantities in the African elephant compared to bovine milk. Moreover, despite the absence of α-caseins, milk of African elephant still contains casein micelles (Madende *et al.,* 2018).

1.3.3. Milk carbohydrates

1.3.3.1. Mammalian milk carbohydrates

Lactose is the main carbohydrate in the milk of most mammalian species, although milk of marsupials, monotremes, and some eutherians contain little lactose (Jenness *et al.,* 1964; Messer & Mossop, 1977). Oligosaccharides are carbohydrate components that are also found in the milk of most mammals. These carbohydrate compounds are molecules composed of a small number of monosaccharide units that can exist in a linear or branched form (Urashima *et al.,* 2004). Oligosaccharide composition in milk of different mammalian species is distinct (Urashima *et al.,* 2001). Both human and elephant milk show considerably high oligosaccharide concentrations compared to species such as cow, pig, horse, and rhesus monkey (Kunz *et al.,* 1996). The milk of species such as the Southern elephant seal and the bear mainly consists of oligosaccharides and the total concentration is also low (Jenness *et al.,* 1972; Carlini *et al.,* 1994). Human milk contains 130 oligosaccharides and is divided into 12 groups based on their core structures (Haeuw-Fievre *et al.,* 1993; Newburg & Neubauer, 1995). However, human milk is distinct from other eutherian species due to the complex sialylated and fucosylated oligosaccharides that occur at high concentrations (Urashima et al, 2012b).

Lactose is a disaccharide synthesized within a lactating mammary gland, a transgalactosylation reaction involving the transfer of the uridine diphosphate galactose (UDP-Gal), a donor molecule, to a glucose acceptor molecule catalyzed by lactose synthase, an enzyme complex consisting of β4-galactosyltransferase and α-lactalbumin. The oligosaccharide biosynthesis involves the β4-galactosyltransferase, where galactose is transferred from the uridine diphosphate galactose (UDP-Gal) to non-reducing N-acetylglucosamine (GlcNAc) residues, thus forming N-acetyllactosamine (Gal(β1-

4)GlcNAc) units. The preferred acceptor is changed from N-acetylglucosamine (GlcNAc) to glucose by α-lactalbumin in its presence, its expression is, therefore, the key to lactose present in milk (Rajput *et al.,* 1996; Urashima *et al.,* 2012a).

1.3.3.2. African elephant milk carbohydrates

The carbohydrate composition of the African elephant milk (*Loxodonta africana*) consists of two major sugars and these are lactose and oligosaccharides. A major oligosaccharide present in the milk of the African elephant was reported to be isoglobotriose (Gal(α1–3)Gal(β1–4)Glc) (Osthoff *et al.,* 2005). The milk of species such as the Asian elephant, polar bear, the coati, and the giant panda also contain isoglobotriose (Kunz *et al.,* 1999; Urashima *et al.,* 1999a, 2000; Nakamura *et al.,* 2003; Uemura *et al.,* 2006).

Based on the studies done by Uemura et al. (2006), the Asian elephant milk contains 1 neutral oligosaccharide and 10 sialyl oligosaccharides. The high ratio of sialyl oligosaccharides reported in the oligosaccharide fraction of elephant milk may be of importance, especially in the development of brain components of the suckling calves (Messer & Urashima, 2002). The majority of the acidic milk oligosaccharides contain N-acetylneuraminic acid (Neu5Ac) or N-glycolylneuraminic acid (Neu5Gc), while sialyl oligosaccharides only contain N-acetylneuraminic acid (Neu5ac). The type I (Gal(β1-3)GlcNAc) and type II branch (Gal(β1-4)GlcNAc) were observed in the Asian elephant milk, but the type II branch (Gal(β1-4)GlcNAc) was reported to be the dominant oligosaccharide branch (Kunz *et al.,* 1999). However, human milk has the type I chain (Gal(β1-3)GlcNAc) which is more prominent than the type II chain (Gal(β1-4)GlcNAc).

The prominence of the type I branch in human milk may be related to the growth of specific intestinal microflora (Urashima *et al.,* 2012a).

Osthoff et al. (2008) reported that African elephant milk, at 4 days after birth, contained a variety of neutral and sialyl oligosaccharides. Some of the oligosaccharides observed in the African elephant milk are present in the milk of other mammals. These include the short oligosaccharide Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)Glc (La 1.1.1 and La 1.1.2), which is found in human milk and platypus milk (Kobata & Ginsburg, 1969; Amano *et al.,* 1985; Martin-Pastor & Bush, 2000; Sumiyoshi *et al.,* 2003). Another oligosaccharide shared with human milk is Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)Glc (La1.1.3) (Yamashita *et al.,* 1977). The neutral oligosaccharide Gal(α1–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)Glc (La 1.1.4 and La 1.1.8) is present in the Japanese black bear, polar bear and the Ezo brown bear (Urashima *et al.,* 1999b; Messer & Urashima, 2002). Two neutral oligosaccharides, Gal(α1–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)Glc and Gal(α1–3)Gal(β1–4)[Fuc(α13)]GlcNAc(β1–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)Glc contain the α-Gal epitope (Gal(α1–3)Gal(β1–4)GlcNAc-R).

African elephant milk contains short acidic oligosaccharides Neu5Ac(α2–3)Gal(β1–4)Glc (La1.2.1, 3'-SL) at high concentrations. These oligosaccharides are present in the milk of multiple mammalian species. The oligosaccharide with chain Neu5Gc(α2–3)Gal(β1–4)Glc (La 1.2.2.b) lacks the N-acetylneuraminic acid, instead, it contains the N-glycolylneuraminic acid structure, the oligosaccharide is also present in bovine and ovine colostrum (Veh *et al.,* 1981; Nakamura *et al.,* 1998). The Neu5Ac(α2–3)Gal(β1–4)[Fuc(α1–3)]Glc (La 1.2.3 and La 1.2.4) oligosaccharide were also observed in human, Asian elephant and giant panda milk (Grönberg *et al.,* 1989; Nakamura *et al.,* 2003;

Uemura *et al.,* 2006). Neu5Ac(α2–3)Gal(β1–4) [Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)Glc (La 1.2.7) contains a terminal sialyl Le^x structure and is also present in Asian elephant milk (Uemura *et al.,* 2006). Sugars that have a sialyl Le^x were reported to be analogues of the selectin ligand, which makes it possible for a sugar to function as a colonic anti-inflammation factor (Vestweber & Blanks, 1999). Acidic oligosaccharides Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)[Fuc(α1–3)]Glc (La 1.2.6) and Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal(β1–4)Glc (La 1.2.9.b) are present in human milk, but absent in Asian elephant milk (Smith *et al.,* 1987). The Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)[Gal(β1–4)GlcNAc(β1–6)] Gal(β1–4)Glc (La 1.2.8) and Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3){Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc (La 1.2.9.a) are also present in human milk but absent in Asian elephant milk (Grönberg *et al.,* 1989). The oligosaccharide Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc (La 1.2.5) is present in Asian elephant milk (Kunz *et al.,* 1999; Uemura *et al.,* 2006). Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–3){Gal(α1–3)Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)}Gal(β1–4)Glc (EM 59) is present in the milk of mink, Asian elephant and Japanese black bear (Urashima *et al.,* 2004, 2005; Uemura *et al.,* 2006).

The oligosaccharide composition of the African Elephant milk consists of the type II chain. Both the African and Asian elephant oligosaccharides contain both non-reducing α(2–3)-linked Neu5Ac and non- reducing α(2–6)-linked Neu5Ac (Messer & Urashima, 2002; Osthoff *et al.,* 2008). The oligosaccharides that are fucosylated in both the African and Asian Elephants consist of the Fuc(α1–3) residue only.

Lactation has an effect on the carbohydrate composition of African elephant milk. A decrease in lactose and an increase in the oligosaccharide content with advancing lactation were observed (Osthoff, 2012). Therefore, the reported data suggest that fat

replaces lactose as the principal energy source at mid-lactation (approximately 15 months) (Osthoff, 2012). The sugar content of the African elephant milk is 3.7 %, which is a bit low compared to the Asian elephant having 4.0 – 8.4 % carbohydrate content in its milk (McCullagh & Widdowson, 1970). Osthoff et al. (2005, 2007a) reported a decrease in the lactose content of the African elephant milk from 5.3 to 1.2 % at 4- and 47-days post-partum and at twelve to eighteen months postpartum, a decrease from 3.9 to 0.7 %. An increase in the oligosaccharide content was also observed from 1.2 to 1.5 % at 4- and 47-days post-partum and a decrease from 2.6 to 1.1 % at 12 to 18 months postpartum, thus making the oligosaccharides major sugars in the milk of the African elephant approximately 14 months.

1.3.4. Minor milk nutrients

1.3.4.1. Mammalian milk minerals

Milk components are responsible for the efficient growth of the neonate and these include fats, proteins, sugars, vitamins, and minerals. Minerals form part of a small fraction of milk and contain all the essential mineral elements required for growth (Bates & Prentice, 1996). The minerals and ions found in milk include calcium, sodium, magnesium, potassium, inorganic phosphate, and chloride. These mineral elements have key functions in milk, such as maintaining milk pH, ionic strength and osmotic pressure (Zamberlin *et al.,* 2012). Other mineral components bind to milk proteins, thus maintaining structure, stability and optimizing protein functions, and some minerals are involved in the oxidation of lipids (Fox *et al.,* 2015c). The mineral composition of individual mammalian species is unique and this is due to factors such as the lactation stage, infection of the udder, seasonal variations and feed (Gaucheron, 2005).

Mineral elements exist in both the soluble and colloidal phases in milk. The mineral content has an effect on the properties of milk; they can influence the gelation and sedimentation properties as well as the susceptibility of milk to rennet (Tsioulpas *et al.,* 2007). Calcium and other divalent ions in the serum can influence the environment surrounding the negatively charged casein micelles (Horne & Parker, 1981).

Calcium and phosphorus are principal mineral elements in milk. In bovine milk, the majority of the calcium occurs in the skim milk fraction, two-thirds of this exists as calcium phosphate in the colloidal phase and the remaining calcium occurs in the soluble fraction (Zamberlin *et al.,* 2012).

1.3.4.2. Elephant milk minerals

The mineral content of the African elephant milk is more or less the same as that in cow's milk, the difference being the high levels of potassium found in elephant milk (McCullagh & Widdowson, 1970). In African elephant milk, the phosphorus content increases with advancing lactation, which may be directly correlated to the protein content, that also increases with progressing lactation time (McCullagh & Widdowson, 1970). The Asian elephant has high amounts of phosphorus and changes of phosphorus and calcium were reported with advancing lactation (Abbondanza *et al.,* 2012). Moreover, the phosphorus content of cow and goat milk is higher than the amount reported by McCullagh and Widdowson (1970) for African elephant milk (Ceballos *et al.,* 2009).

1.3.4.3 Mammalian milk vitamins

Vitamins are a group of organic micronutrients that are required for normal growth, maintenance and proper functioning of animal bodies (Gao *et al.,* 2008). In addition, vitamins play other significant roles such as promoting the health of the skin, hair, eyes, nervous system, mouth, and liver and maintaining muscle tone of the digestive tract lining (Fenech, 2001; Gao *et al.,* 2008). The vitamins found in milk include the fat-soluble (A, D, E, and K) and water-soluble (the B vitamins, vitamin C and folate) vitamins (Fox *et al.,* 2015d). Individual vitamin components vary in concentration in each species milk due to diet, stage of lactation and breed.

In bovine milk, vitamin A exists as retinol, retinol esters and carotenes (Fox *et al.,* 2015d). The content of vitamin A and other vitamins such as riboflavin, vitamin $B_{6,}$ and pantothenic acid in milk are dependent on the diet, breed and season (Scott & Bishop, 1986; Indyk *et al.,* 1993). In addition, vitamin C levels in milk are reduced by handling and storage. It has been reported that season variation also has an impact on vitamin C concentrations (Fox *et al.,* 2015d). β-Carotene in goat milk is converted to vitamin A, thus making the milk whiter than bovine milk (Park *et al.,* 2007). Vitamin E in milk exists as α-tocopherol and the concentration is dependent on the fat content of the milk (Fox *et al.,* 2015d). Moreover, niacin occurs as nicotinamide in milk and its content is moderately affected by breed, diet, lactation and season variation (Jenness, 1988).

Vitamin A occurs in high amounts in goat, sheep and buffalo milk than in bovine milk (Narayanan *et al.,* 1952; Park *et al.,* 2007). The riboflavin and vitamin B_6 content in Asian elephant milk is approximately similar to that found in bovine milk (Markuze, 1939; Peters *et al.,* 1972). In addition, the milk of camel, mare and Asian elephant

contain higher amounts of vitamin C than bovine milk (Markuze, 1939; Csapó *et al.,* 1995; El-Agamy & Nawar, 2000). The vitamin B levels in both bovine and sheep milks are due to rumen synthesis (Haenlein, 2004). Vitamin K content in bovine and human milk is present in low amounts *(*Haroon *et al.,* 1982; Fox *et al.,* 2015d).

It is worth noting that no data is available in the literature regarding vitamins in African elephant milk. These are some of the short-comings the current study is attempting to address.

1.3.4.4. Mammalian milk sterols

Sterols are a group of compounds that occur naturally in animals, plants, and fungi, and can be synthesized by some bacteria (Laakso, 2005; Wei *et al.,* 2016). There are different types of sterols in nature and they exist in various forms such as esters with fatty acids, cinnamic acid, and ferulic acid, or can exist as free sterols (Piironen *et al.,* 2000; Moreau *et al.,* 2002). Sterols have multiple functions such as maintaining membrane fluidity, cell signaling, general metabolism and stress tolerance (Lampe *et al.,* 1983; Wei *et al.,* 2016).

Cholesterol is a major sterol component in most mammalian milk, making up at least 95 % of total sterols (Fox *et al.,* 2015a). Other sterols such as beta-sitosterol, delta-4-cholesten-3-one, lanosterol, delta-3,5-cholestadiene-7-one, dihydro-lanosterol, and 7-dehydrocholesterol have been identified and characterised in ruminant milk (Jensen, 2002). The majority of the cholesterol in bovine milk is located in the milk lipid globule membrane (MLGM) (Fox *et al.,* 2015a). Some of the cholesterol in milk is bound to proteins specifically the β-lactoglobulin (Wang *et al.,* 1997). Moreover, approximately 10 % of cholesterol in bovine milk is esterified (Jensen, 2002).

The unsaponifiable matter in mare milk is higher than cow, goat, Asian elephant and human, and cholesterol had been reported to be the main sterol component in these milk lipid fraction (Peters *et al.,* 1972; Posati *et al.,* 1975; Malacarne *et al.,* 2002). Among the aforementioned species, goat milk has the lowest amount of cholesterol (Posati *et al.,* 1975). The low levels of cholesterol in goat milk is of importance to human nutrition since cholesterol is associated with cardiovascular diseases (Haenlein, 2004). However, the majority of cholesterol in caprine and bovine milk is in a free state (Jenness, 1980). In addition, the caprine fatty acid profile of cholesterol ester has been shown to contain more oleic and palmitic acids fractions than bovine (Park *et al.,* 2007).

There is no literature available on sterols in African elephant milk thus far and the current study is attempting to address the short-coming.

1.4. Colostrums

Colostrum is a sticky white or yellowish nutrient-rich liquid produced immediately after the birth of a mammalian calf. Colostral composition differs greatly from normal milk (Blum & Baumrucker, 2002; Ontsouka *et al.,* 2003). This nutrient-rich fluid contains a higher amount of protein, immunoglobulins, growth factors, fat, vitamins, ash, bioactive molecules and antimicrobial peptides than mature milk (Blum & Baumrucker, 2002; Uruakpa *et al.,* 2002). There are marked differences between colostrum and normal milk but, in some species the changes in the composition are small. Species such as humans, rabbits, and baboons with prenatal passive immunization show small changes in some of the milk components (Langer, 2009). In ungulates, a large difference is observed in the composition of colostrum and mature milk, especially in the protein content.

Therefore, the difference in both colostrum and milk composition reflects the diverse species-specific strategies used by eutherians to transfer passive immunity (Langer, 2009). The elephant colostrum was described as a three-layered fluid, with a creamy top layer, the blue layer, and a yellow stratum, which consists mostly of mucous cells (Doremus, 1882). The colostral period lasts for 5-7 days after parturition (Blum *et al.,* 2002).

Colostrum consists of two primary components: immune and growth factors. Immune factors play a significant role in protecting calves from yeast and fungus, viruses and bacteria (Thapa, 2005). Immune factors also protect the mammary gland of the host from pathogenic organisms (Stelwagen *et al.,* 2009). Moreover, immunoglobulins are a major colostrum component; they make up 5 % of the colostrum content (Stelwagen *et al.,* 2009). This is because young calves are born without blood immunoglobulins and therefore, depend on the colostrum for immune components (Abd El -Fattah *et al.,* 2012). Therefore, the transference of passive immunity throughout the colostral period is essential for the infant's health and survival during its first days after parturition. In addition, growth factors are responsible for stimulating growth of the neonate (Thapa, 2005).

1.5. Milk analysis methods

The analytical methods applied in milk composition determination are subject to quality control that is strict, which includes collaborative interlaboratory studies that evaluate assay performance and the viability of alternative methods (Barbano *et al.,* 1988). However, this is not true for the analysis of milk produced by mammals other than dairy mammals, where various analytical methods have been utilized without method

validation or standardization (Oftedal & Iverson, 1995). The methods or procedures used for the analysis of cow milk may not be effective for milk of other mammals especially the non-dairy ones. The techniques used for the analysis of milk components may give diverse responses due to the differences in the composition and structure of these components (Oftedal and Iverson, 1995; Oftedal *et al.,* 2014).

Proteins have been studied for over 50 years and questions concerning the expression, structure, and modification of milk proteins remain unanswered (O'Donnell et al, 2004). Different methods for protein analysis have been developed over the years and these include the; Bradford, Dumas, biuret, Fourier transform infrared spectroscopy, Lowry, macro-Kjeldahl, micro-Kjeldahl, and nesslerization (Oftedal *et al.,* 2014). Many established methods for protein analysis require time, expertise and large sample volumes, therefore alternative methods are desired, especially micro-methods that can accommodate small milk samples from small species such as rodents and other mammals at remote locations that are hard to sample (Arnould *et al.,* 1995; Hood *et al.,* 2006). The carbon-hydrogen-nitrogen (CHN) gas analysis, is one attractive method which requires a small sample size (Oftedal *et al.,* 2014). This is an improved Dumas method of analysing nitrogen.

Traditional methodologies or techniques for fat determination involve the use of organic solvents for extraction, drying of the extract and gravimetric determination of fat. The methods include the Folch, Weibull-Berntrop, Soxhlet, and the Röse-Gottlieb methods (Shin & Park, 2015). The most reliable methods for quantitatively extracting lipids from a variety of animal tissue types is the Folch and Bligh-Dyer method (Bligh & Dyer, 1959; Folch *et al.,* 1957). The Folch method has been one of the commonly used methods in studies relating to fat extraction, and this is due to its mild working conditions, which do

not require high temperature nor pressure (Pérez-Palacios *et al.,* 2008). The Soxhlet method is recommended for the estimation of the fat content. The disadvantages of the Soxhlet method are that it has a relatively long extraction time and also requires high temperatures. The automated version of the Soxhlet method, on the other hand, offers several advantages over the original method, such as decreased extractant volume, shorter extraction time, and simultaneous extraction of various samples (Luque de Castro & Priego-Capote, 2010). The AOAC 996.06 method is used to analyse the total, saturated, polyunsaturated, and monounsaturated fats. It is a commonly accepted method due to its sufficient accuracy and repeatability (Ngeh-Ngwainbi *et al.,* 1997). The aforementioned method involves the extraction of triacylglycerols and fatty acids from a food sample, which are then methylated to fatty acid methyl ester using BF_3 in methanol. Gas chromatography is used to quantitatively measure the fatty acid methyl esters (FAME's). The amount of all individual fatty acids expressed as triglyceride equivalents make up the total fat (Shin & Park, 2015).

The method devised by Dubois and co-workers is ideal for direct determination of sugars in milk (Dubois *et al.,* 1956). The phenol-sulphuric acid method can be used for the quantitative colorimetric micro-determination of carbohydrates and its methyl derivatives, polysaccharides, and oligosaccharides (Dubois *et al.,* 1951). The method is easy, rapid and sensitive, and gives reproducible results. For the structural elucidation of sugars and their derivatives, the nuclear magnetic resonance spectroscopy (NMR spectroscopy) method is used (Duus *et al.,* 2000). The technique is used for the identification of individual carbohydrates or sequences of residues and can be used to identify specific sugars or structural motifs and the composition of linkages found in relevant databases such as the Carb Bank or SUGABASE (Vliegenthart *et al.,* 1983; Duus *et al.,* 2000). The method is one of the most important techniques and probably the

most often used analytical technique since it provides indispensable information about sugars (Duus *et al.,* 2000).

1.6. Discussion and Conclusions

Milk is a complex fluid that is composed of multiple macronutrients such as protein, fat carbohydrates, and micronutrients such as minerals, vitamins, and a whole array of organic acids and amines. The composition of these nutrients differs within species and the alteration may be due to nutrition, genetic factors as well as the stage of lactation. The major sugar in the milk of many species is lactose, while oligosaccharides are the dominant sugar in the milk of other species such as the sea lion, humans, and African Elephant. The fatty acid profile of most mammals is mainly composed of long-chain and unsaturated fatty acids, while African elephant contain medium-chain saturated fatty acids. With regards to the casein content of milk, bovine milk is composed of all four caseins (α_{s1}-, α_{s2}-, β- and κ-casein), whereas some mammalian species are devoid of some of the caseins, for example, human milk is devoid of α_{s2}-casein.

The nutritional parameters of African elephant milk are different from all the mammals that have been studied. The milk carbohydrates have a high oligosaccharide content and unique branch chains have been reported (Osthoff *et al.,* 2008). The proteins only consist of β – and K- casein (Madende *et al.,* 2015, 2018). Lauric and capric acid are found in high amounts in the fat and the fatty acid composition was shown to change with advancing lactation (Osthoff *et al.,* 2005, 2007a). The reason for the African elephant's distinct milk composition may be genetic. This is due to the fact that, taxonomically, the African elephant falls in a clade called the Atlantogenata, specifically

the Afrotheria lineage, which split early from the clade Euarchontoglires, after the infraclasses Placentalia and Marsupialia have separated (Murphy *et al.,* 2001).

The milk of the African elephant has been studied and the composition has been described. Some aspects about the African elephant milk composition have been reported in great detail and changes in the composition over lactation have been pieced together from different elephant sub-species and individuals. There is still insufficient information on the milk composition due to gaps of weeks or months between data points.

1.7. Aims of the study

The aim of the research was to study the milk composition of the African elephant over a full lactation period.

The objectives of the study were:

1. To study the changes of the macronutrient composition (fat, protein, carbohydrates) and energy value of African elephant milk over lactation.

2. To study the changes of micro-nutrient composition (minerals, vitamins, and sterols) of African elephant milk over lactation.

3. To study the changes of fatty acid composition of African elephant milk over lactation.

4. To study the changes of oligosaccharide composition of African elephant milk over lactation.

Chapter 2

MATERIALS AND METHODS

2.1. Introduction

Milk is a complex fluid that has been constantly studied since the beginning of the 19th century. The composition and some properties of its various components are now well understood (Tremblay *et al.,* 2003). Many analytical techniques that were used to measure the composition of milk and dairy-related products have been adapted, altered or changed over the last 15 to 20 years. Moreover, analytical techniques are subject to stringent quality control measures which include interlaboratory collaborative studies that evaluate assay performance and the viability of different analytical techniques (Barbano & Lynch, 2006). Although standardized techniques are normally applied as analytical procedures of milk from non-dairy species and wild mammals, other techniques have also been employed without method validation or standardization, which makes the comparison of milk data difficult (Oftedal & Iverson, 1995).

Milk composition varies greatly amongst mammals and variation is brought by different factors such as lactation, genetic factors, seasonal variation and diet (Jenness, 1988). The differences such as very high or low nutrient contents may have an effect on the performance of analytical techniques. Methods used for the analysis of cow milk may not be effective for milks of other mammals especially the non-dairy ones (Oftedal *et al.,* 2014). Therefore, analytical techniques that are validated for milk from dairy species may or may not be suitable for other mammalian milks, depending on assay sensitivity

to structural differences in particular milk components (Oftedal & Iverson, 1995). It may therefore, be necessary to employ more than one technique to compensate for errors.

Milk fat is a major component of energy and consists of a mixture of complex fatty acids. Generally, lipids are soluble in organic solvents such as ether and chloroform but not in water. Therefore, the properties mentioned above are used as a basis to separate lipids from other components such as water, carbohydrates, and proteins in milk (Min & Ellefson, 2010). The Röse-Gottlieb and Folch method were used in the current study to extract the total milk fat, followed by esterification and gas chromatography to measure the fatty acid methyl esters (FAME's) of African elephant milk. The Röse-Gottlieb technique has long been considered the reference method in studies relating to the extraction of fat in milk (Oftedal *et al.,* 2014). Moreover, the procedure involves a mixture of petroleum ether and ethyl ether in a Mojonnier flask and the fat extracted is dried to a constant weight and expressed as percent fat by weight (Min & Ellefson, 2010). The Folch method is another procedure commonly used to extract fat due to its mild working conditions, which do not require high temperature nor pressure (Pérez-Palacios *et al.,* 2008). In addition, the procedure involves chloroform and methanol and allows extraction of fat for subsequent fatty acid examination (Budge *et al.,* 2006). However, method performance may be different for milks with a high-fat content (Oftedal *et al.,* 2014).

The milk protein component is composed of a wide variety of proteins which are mostly synthesized in the mammary gland (Farrell *et al.,* 2004). These can be categorized into different groups namely the caseins, whey and milk fat globule membrane (MFGM) (D'Alessandro *et al.,* 2010). Various methods exist for the estimation of protein in milk (Oftedal *et al.,* 2014). In the current study, only the macro-Kjeldahl and the Dumas

methods were used to measure the protein content and composition in the African elephant milk. The macro-Kjeldahl is one of the reference methods used for the determination of protein in milk and dairy products (Tremblay *et al.,* 2003). This technique makes use of acid to digest organic compounds so that nitrogen can be released and estimated by an appropriate titration. Moreover, this analytical technique has been studied thoroughly and accounts for over a thousand publications (Tremblay *et al.,* 2003). The Dumas method is one of the attractive alternative techniques whose basis involves the conversion of all forms of nitrogen in the sample to nitrogen oxides in the combustion chamber. The reduction chamber reduces all oxides to nitrogen gas and measurement is done either volumetrically or via thermal conductivity (Sweeney & Rexroad, 1987). In addition, a conversion factor of 6.34 is used to convert the nitrogen of the sample to protein content (Chang, 2010).

Milk contains different types of carbohydrates namely lactose, oligosaccharides, and other monomer constituents (Jenness *et al.,* 1964). Therefore, for the determination of these carbohydrates, procedures such as the phenol-sulphuric acid, Somogyi–Nelson method and high-performance liquid chromatography are used (HPLC) (BeMiller, 2010). In the current study, only the phenol-sulphuric acid and HPLC were employed, similar to a study by Osthoff et al. (2005, 2007a, 2008). The phenol-sulphuric acid method involves dehydrating glucose to hydroxymethylfurfural in an acid medium. This forms a yellow-brown substance with phenol which has an absorption maximum at 490nm (Dubois *et al.,* 1956). The alternative HPLC method offers qualitative analysis with peak integration and quantitative analysis of milk sugars by means of standards (BeMiller, 2010).

While the quantification of lactose may be carried out by several chemical spectrophotometric or chromatographic methods, the quantification of oligosaccharides

provide challenges (Cataldi *et al.,* 2003; Chávez-Servín *et al.,* 2004). It specifically involves the N-neuraminic acid side chains of the acidic oligosaccharides, because they contribute to the mass, but are not detected by the standard carbohydrate dedicated methods. As a result, the best method for lactose quantification is by chemical labelling, separation by HPLC and quantification of each oligosaccharide (Sumiyoshi *et al.,* 2003).

The energy content in milk is generally calculated from its proximate components with the aim of measuring the rate of energy transfer from mother to infant during lactation. Methods often used to assess the energy content of milk is the bomb calorimetric method and results from reference procedures (Oftedal *et al.,* 2014). In addition, gross energy levels in milk may be predicted using factors derived by Perrin (1958) which are applied to all mammalian milk.

Minor compounds such as vitamins, minerals, and sterols form part of a small fraction of milk. Different analytical methods exist for the determination of minor nutrient compounds in milk. The HPLC is used for the analysis of vitamins in milk. Moreover, the analysis of minerals is achieved by ashing and quantification by inductively coupled plasma optical emission spectroscopy (ICP-OES). Lastly, sterol analysis involves a three-step process namely, basic digestion of sample, organic extraction, derivatization, and quantification by gas chromatography.

2.2. Materials and Methods

2.2.1. Sample collection and preparation

The research adhered to the following rules and standards: ethics clearance, ECUFS NR 193/2015; section 20 permission UFS-AED 2016/0106. Milk samples were obtained from three free-roaming African elephants at different lactation stages. Elephant one (Bela), provided milk for the complete lactation, day one up to 19 months. This elephant lived in Pamuzinda Safari Lodge in the Mashonaland central province, Zimbabwe. Cites permit numbers 164211 and 199643 were obtained for the importation of African elephant milk from Zimbabwe to South Africa. Milk samples from elephants two (Mussina) and three (Shan) roamed in the Adventures with elephant's reserve, Bela Bela, Limpopo province, South Africa, and provided milk from 11 to 14 months and 21 to 23 months of lactation respectively. Milk was drawn from the elephant cows at weekly intervals (Bela) during the first month and two-weekly intervals thereafter. Analyses were carried out on every sample of the first month of lactation (Bela) followed by one sample of each month. Milk samples of large volume (20 – 50 ml) were selected so that every parameter could be determined of the same sample. However, in a few cases, milk samples of smaller samples (less than 10 ml) of the next collection interval had to be used. The elephants were tame and milk could be obtained without tranquilization by palpation of the teats. The elephant milk samples were stored frozen and kept at -23^{o} C during transportation. Once in the laboratory, milk samples were thawed at 39^{o}C in a warm water bath with gentle swirling, sub-divided in appropriate volumes for individual analytical procedures and re-frozen. This was done to keep subsequent thawing and re-freezing steps at a minimum. Due to the complexity and expense, the analysis of vitamins, oligosaccharides by Biogel P2 chromatography and fatty acid analysis were carried out on single samples but were followed by a second if large deviations were suspected. The determination of nitrogen, saccharides by chromatography, minerals,

and sterols were carried out in duplicate, and energy in triplicate. The data was presented as averages of two or three.

2.2.2. Density Calculation

For the determination of density all measurements were done at constant temperature and pressure. The elephant milk was thawed at 39^{o} C and 5 ml of the sample was weighed. The weight (g) obtained was used to calculate the density of African elephant milk (Oguntunde & Akintoye, 1991).

The following formula was used to calculate density:

$$\rho = \frac{m}{V}$$

Where,

m = mass in g of weighed sample.

V = volume in ml of sample.

2.2.3. Moisture and Mineral Analysis

Analysis of minerals was achieved by ashing and quantification by inductively coupled plasma optical emission spectroscopy (ICP-OES). The methods used in this study were performed according to international dairy federation Standards 154 (1992) and 156A (2000) (International Dairy Federation Standard, 1992, 2000).

Crucibles were dried in a hot air oven and weighed. Approximately 0.5 g elephant milk was weighed in a crucible. The crucible was heated three subsequent times in an oven

at 102^{o}C for 60 minutes each until a constant mass was obtained. The mass of the dry milk sample was used to determine the moisture content. The dried milk sample was then incinerated in a muffle furnace at 550 ^{o}C for approximately 2 hours. Nitric acid (2 parts water to 1-part nitric acid) was added to each ash sample and returned to the furnace to complete the digestion until white ash was obtained. The ash was dissolved in the above nitric acid solution and transferred to a 50 ml volumetric flask. The crucible was rinsed out three times with the same nitric acid solution into the 50 ml volumetric flask. Distilled H_2O was used to make up to 50 ml. Minerals were analysed by ICP-OES, by the Center of Groundwater Studies, University of the Free State, according to APHA method 3120 B (2005).

2.2.4. Vitamin Analysis

Analyses of vitamins A, D_3, E and K were carried out by Merieux NutriSciences, Swft Silliker (Pty) Ltd, Claremont, South Africa. An Eagle Biosciences Vitamin HPLC assay kits were used; VAE 31-H100 for vitamins A and E (Comstock *et al.,* 1993; Sushil *et al.,* 1994), VD331-H100 for vitamin D3 and VK131-H100 for vitamin K (Hodges *et al.,* 1993; Szulc *et al.,* 1994; Friedrich, 1988). Reagent and sample preparation and HPLC analyses were performed as described in the procedure.

2.2.5. Carbohydrates Analysis

2.2.5.1. Analysis of mono-, di- and trisaccharides by HPLC

Saccharides were extracted from milk by adding 500 µl 25 % Trichloroacetic acid (TCA) to 1 ml milk sample. The mixture was vortexed for 5 seconds, transferred to Nanosep 3K MF Centrifugal Devices (Pall Life Sciences, Michigan, USA) and centrifuged in an

Eppendorf centrifuge (Bio-Rad, South Africa) for five minutes at 13000 rpm. The filtrate was subjected to HPLC analysis.

A Waters Breeze HPLC system was used to determine the carbohydrate content of the filtrate by means of Biorad Aminex 42C (3007.8mm) and Water Sugar Pak 1 (3007.8 mm) columns at 84 ^{o}C with a differential refractive detector. Deionized water was used as the mobile phase. The mobile phase eluted at 0.6 ml/min. Quantification was done using maltotriose, lactose, glucose, and galactose as standards and isoglobotriose prepared from African Elephant milk (Osthoff *et al.,* 2008; BeMiller, 2010).

2.2.5.2 Separation of saccharides by chromatography

Mono-, di-, tri- and oligosaccharides were separated as described by Osthoff et al. (2008). The elephant milk was thawed at 39^{o} C and 1.5 ml was extracted with four volumes of chloroform/methanol (2:1, v/v). The mixture was agitated and the emulsion was centrifuged at 6450 *x* g, 4 ^{o}C for 30 minutes. The lower chloroform layer and the denatured protein with other unwanted milk contents were separated. The methanol from the upper layer was evaporated and the lyophilized residue was designated as the carbohydrate fraction.

The carbohydrate fraction was dissolved in 2 ml distilled water and 400 µl samples were passed through a Bio-Gel P-2 (<45µl, Bio-Rad, USA) column (1.5 x 90 cm, void volume =70 ml). The column had been calibrated with galactose, glucose, lactose, and dextran.

The Elution with distilled water at a flow rate of 6 ml/h and fractions were collected every 20 minutes (2 ml fractions). Aliquots (40 µl) of each fraction were analysed for hexose with the phenol-H_2SO_4 method (Dubois *et al.,* 1956). 40 µl of each fraction was micropipette into a separate test tube, followed by 200 µl of 5 % phenol solution and 1 ml of concentrated sulphuric acid. The tubes were allowed to stand for 10 minutes and then shaken and placed in a water bath at 25-30 ^oC for 10 to 20 minutes. The absorbance of each sample was measured at 490 nm. Peaks containing unabsorbed materials were pooled and lyophilized.

Each fraction was dissolved in 1500 µl of 50 mM Tris-hydroxyaminomethane-HCl buffer (pH 8.7) and200 µl, 400 µl and 500 µl respectively were loaded and subjected to anion exchange chromatography using a DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Sweden) column (0.9 x 45 cm, void volume = 16 ml). Elution was with the same Tris buffer at a flow rate of 6 ml/h and fractions of 2 ml were collected. After all three runs, the adsorbed components were eluted using a linear gradient of 0-0.5 M NaCl in the buffer. Aliquots were analysed for hexose as above. Barely any components were found in the fractions eluted by the salt gradient.

2.2.5.3. Quantification of carbohydrates

Quantification of monosaccharides, lactose, and isoglobotriose was carried out by integration of HPLC chromatogram peaks. For the quantification of oligosaccharides, the areas under peaks of Bio-Gel P2 chromatograms were calculated. The lactose peak of the latter was assigned the HPLC-obtained concentration of the same sample, and the oligosaccharide content calculated relative to that of the lactose.

2.2.6. Protein Analysis

2.2.6.1. Kjeldahl Method

Nitrogen and Protein were determined according to the AOAC. 2005. A nitrogen conversion factor of 6.38 was used to convert nitrogen to protein.

2.2.6.2. Dumas/Leco

2.2.6.2.1. TN (Total nitrogen) Determination

A sample volume of 100 µl was placed in an Eppendorf tube. The Total Nitrogen (TN) was determined using the LECO combustion analysis. The non-protein nitrogen (NPN) percentage was subtracted from the TN percentage for the total protein percentage (Sweeney & Rexroad, 1987).

2.2.6.2.2. Protein Fractionation

Non-protein and Whey protein, samples were fractionated by selective precipitation according to the method of Csapó et al. (1996).

2.2.6.2.3. Non-protein Nitrogen determination

A volume of 200 µl of 15 % trichloroacetic acid (TCA) was added to 100 µl sample. The mixture was vortexed for 5 seconds and then centrifuged in an Eppendorf centrifuge (Bio-Rad, South Africa) for 5 minutes at 7000 rpm. The supernatant was collected and the NPN was determined by using the LECO combustion analysis. The nitrogen percentage was obtained and multiplied by a dilution factor of 3 to calculate the percentage of nitrogen.

2.2.6.2.4. Whey Protein Determination

A volume of 100 µl dH_2O was added in 100 µl of milk sample. The mixture was vortexed for 5 seconds. Following complete mixture, 30 µl of acetic acid (AcOH) was added and the mixture was vortexed for 5 seconds. The mixture was allowed to stand for 20 minutes after which 250 µl dH_2O was added to the mixture and the mixture was vortexed for 5 seconds. The mixture was allowed to stand for 1 hour and centrifuged. The supernatant was collected and the whey and NPN percentage were determined using LECO combustion analysis. Whey percentage was determined by subtracting the NPN percentage from the whey and NPN percentage. Casein percentage was then determined by subtracting the whey percentage from the total protein percentage.

2.2.7. Fat and Fatty Acid Analysis

2.2.7.1. Röse-Gottlieb

The Röse-Gottlieb was used for the Extraction of African elephant milk fat, according to IDF Standards 22B 1987 (International Dairy Federation, 1987). A volume of 10 ml milk sample which is equivalent to 10 g of sample was treated with 10 ml concentrated hydrochloric acid in a small beaker. The contents were heated and continuously stirred until the mixture turned dark brown. The mixture was transferred to the Mojonnier fat extraction flask. A volume of 10 ml of ethyl alcohol was added into the mixture before it was later transferred to the fat extraction flask. An additional l 25 ml of ethyl ether was added to the mixture before transfer to the Mojonnier flask. A stopper cork was used to close the flask and the contents were shaken vigorously for 1minute. Following the shaking step, 25 ml of petroleum ether was added and subsequently shook vigorously for 1 minute. The contents were centrifuged at 600 rpm until the upper liquid was clear.

The ether solution was decanted into a suitable flask. The tip and the stopper of the extraction flask were washed with a mixture of equal parts of two solvents and washings were added to the weighing flask. The extraction of liquid remaining in the flask was repeated using 15 ml of each solvent.

The solvent was completely evaporated using a water bath at a temperature that did not cause sputtering. The fat was dried in an oven at 102 ± 2^{o}C to a constant weight. The cooled flask was weighed and fat was removed completely from the container with warm petroleum ether and weighed as before.

The following formula was used to calculate the Fat percentage:

Fat, % (w/w) = $\frac{100\ (W1-W2)}{W3}$

Where,

W_1 = Weight in g of contents in the flask before removal of fat

W_2= Weight in g of contents in the flask after removal of fat

W_3 = Weight in g of material taken for the test

2.2.7.2. Micromethod and Folch extraction of fatty acids

2.2.7.2.1. Lipid extraction

The total lipids from milk samples were quantitatively extracted, according to the method Folch et al. (1957), using chloroform and methanol in a ratio of 2:1. An antioxidant, butylated hydroxytoluene was added at a concentration of 0.001 % to the

chloroform: methanol mixture. A rotary evaporator was used to dry the fat extracts under vacuum and the extracts were also dried overnight in a vacuum oven at 50 °C, using phosphorus pentoxide as a moisture adsorbent. Total extractable fat content (EFC) was determined gravimetrically and expressed as % fat (w/w) per 100 g milk. The fat-free dry matter (FFDM) content was determined by weighing the residue on a preweighed filter paper, used for Folch extraction, after drying. By determining the difference in weight, the FFDM could be expressed as % FFDM (w/w) per 100 g milk. The moisture content of the milk was determined by subtraction (100 % - % lipid - % FFDM) and expressed as % moisture (w/w) per 100 g milk. The extracted fat was stored in a poly top (glass vial, with push-in top) under a blanket of nitrogen and frozen at -20 °C until further analysis.

2.2.7.2.2 Fatty acid analysis

Approximately 10 mg of total lipid from the Folch extraction was transferred into a Teflon-lined screw-top test tube by means of a disposable glass Pasteur pipette. Fatty acids were transesterified to form methyl esters using 0.5 N NaOH in methanol and 14 % boron trifluoride in methanol (Park & Goins, 1994). Fatty acid methyl esters were quantified using a Varian 430 flame ionization GC, with a fused silica capillary column, Chrompack CPSIL 88 (100 m length, 0.25 mm ID, 0.2 µm film thickness). The column temperature was 40–230 °C (hold 2 minutes; 4 °C/minute; hold 10 minutes). Fatty acid methyl esters in hexane (1ml) were injected into the column using a Varian CP 8400 Autosampler with a split ratio of 100:1. The injection port and detector were both maintained at 250 °C. Hydrogen, at 45 psi, functioned as the carrier gas, while nitrogen was employed as the makeup gas. Galaxie Chromatography Software recorded the chromatograms. Fatty acid methyl ester samples were identified by comparing the relative retention times of FAME peaks from samples with those of standards obtained

from SIGMA (189-19). Nonadecanoic acid (C19:0) was used as internal standard. Fatty acids were expressed as the relative percentage of each individual fatty acid as a percentage of the total fatty acids present in the sample. The following fatty acid combinations and ratios were calculated by using the fatty acid data: total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), total polyunsaturated fatty acids (PUFA), total omega-6 fatty acids, total omega-3 fatty acids, PUFA/SFA and omega-6/omega-3 ratio.

2.2.7.2.3. Phospholipid fatty acid analysis

Phospholipids were separated from the extracted lipid fraction with solid phase extraction using silica-bonded NH2 columns with a 500 mg bed mass, 3 ml capacity and 40 µm mesh size obtained from Agilent (Part no. 12102041, MFG code 204107) according to the procedure described by Bossio & Scow (1998). Quantification of total lipids and separation of different fractions was not attempted. Fatty acid methyl esters (FAME) of the phospholipid fraction were prepared for gas chromatography by methylation of the extracted fat, using methanol–BF3 (Slover & Lanza, 1979; Hur *et al.,* 2004; Diaz *et al.,* 2005). Fatty acid methyl esters were quantified using a Varian GX 3400 flame ionization GC, with a fused silica capillary column, Chrompack CPSIL 88 (100 m length, 0.25 mm ID, 0.2 mm film thickness). The column temperature was 40–230 °C (hold 2 minutes; 4 °C/minute; hold 10 minutes). Fatty acid methyl esters in hexane (1 ml) was injected into the column using a Varian 8200 CX Autosampler with a split ratio of 100:1. The injection port and detector were both maintained at 250 °C. Hydrogen, at 45 psi, functioned as the carrier gas, while nitrogen was employed as the makeup gas. Varian Star chromatography software recorded the chromatograms. Fatty acid methyl ester samples were identified by comparing the relative retention times of FAME peaks from samples with those of standards obtained from SIGMA (189-19). Fatty acids were

expressed as the relative percentage of each individual fatty acid as a percentage of the total of all fatty acids present in the sample.

2.2.8. Sterol analysis

Sterol analysis was achieved by a basic digestion, derivatization, and quantification by gas chromatography (Jensen *et al.,* 1991; Alonso *et al.,* 1997).

Approximately 1.0 g of milk sample was divided amongst two glass tubes, where 5µL internal standard (5α- cholestane; 1 µl) and 2 ml of 5M ethanolic potassium hydroxide (KOH) was added. The mixture was vortexed and allowed to saponify in a water bath at 55 ^{o}C for an hour. The contents in the tubes were then cooled to room temperature and 1 ml of distilled water and 3 ml hexane was added. The mixture was vortexed and allowed to stand for phases to separate. The organic top phase was transferred to a clean test tube. The extraction was repeated three times and the organic phases added to the first extraction. The organic matter was dried under nitrogen (N_2) gas on warm sand bath until the volume was sufficiently reduced. The organic matter of the two extracts was combined when transferred into small glass vials. Hexane was used to wash and transfer the organic matter to the vial to complete the drying process.

The extract was dissolved in 100 µl N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heated at 60 ^{o}C for an hour. The BSTFA was evaporated under N_2 gas and the sample was derivatized in 50 µl n-decane.

The sterol derivatives were analyzed by gas chromatography on Varian Factorfour vF5-ms column (30m x 0.32 ID x 0.25 µm film thickness) Helium was used as a carrier gas at a constant flow rate of 3.4 ml/min. Injector temperature at 275 ^{o}C/min. The Column oven temperature programmed as follows: 150 ^{o}C for 1 min ramp at 7 ^{o}C/min to the final temperature of 300 ^{o}C where 1 µl was injected.

2.2.9. Energy Determination

2.2.9.1. Bomb Calorimetry

The general energy content of a subset of milk samples was measured in triplicate in an adiabatic bomb calorimeter (Perrin, 1958).

300 µl of milk sample were added to weighed amounts of cotton wool (50 mg) in calorimeter cups and dried overnight at 60 ^{o}C, to avoid generation of Maillard products, to constant weight. After sample combustion, residual fuse wire and residual acid were measured, with corrections applied to calorimetry data. The energy content of the cotton was subtracted from the total energy measurement to obtain milk gross energy (GE) content.

2.2.9.2. Theoretical calculation

The general energy content was calculated using factors derived by Perrin (1958) for the energy content of milk fat, sugar and crude protein using results obtained from reference methods. The following was used to calculate the Gross Energy (GE)

GE = (9.11kcal/g x %Fat + 5.86 kcal/g x %Protein + 3.95 kcal/g x %Carbohydrate).

Chapter 3

RESULTS

3.1 Introduction

Elephants are one of the largest terrestrial mammals which belong to a taxonomical branch known as the Afrotheria (Murphy *et al.,* 2001). Milk of African elephant is distinct and cannot be compared with the milk of other species. Data on elephant milk has been reported over the past half-century and unique properties are still being discovered. It has been established that elephant milk changes to a great extent during lactation and it's nearly impossible to define a typical milk composition. Moreover, some of the changes in individual milk components are unique to the elephant (Osthoff, 2012). The milk carbohydrates have a high oligosaccharide content and unique branch chains have been reported (Osthoff *et al.,* 2008). The proteins only consist of β – and K-casein (Madende *et al.,* 2015). Lauric and capric acid are found in high amounts in the fat and the fatty acid composition was shown to change with advancing lactation (Osthoff *et al.,* 2005, 2007a).

The majority of the components of African elephant milk have been studied in great detail and changes in composition over the lactation period have been pieced together from different elephant species, sub-species and individuals. However, there is still insufficient information on the milk composition due to gaps of weeks or months between data points (eg., Osthoff *et al.,* 2005, 2007a). The current study seeks to explore the milk composition of African elephant over full term lactation. Milk samples

were obtained from three free-roaming African elephants at different lactation stages. Elephant one (Bela), provided milk for the complete lactation, from day one up to 19 months. Milk samples from elephants two (Mussina) three (Shan) provided milk from 11 to 14 months and 21 to 23 months of lactation respectively for inter-individual comparison. A variety of experimental procedures were utilized in the current study and a majority of these techniques have attracted much success in analyzing the composition of milk, specifically the milk composition of non-dairy species.

3.2. Results

The results of all the analyses are tabulated in Appendix A. The results of each parameter are represented graphically and separately in this section.

3.2.1. Density

The density of the African elephant milk did not change over lactation. The average density was 1.01 ± 0.03 kg/l.

3.2.2. Minerals

Changes in the ash content of African elephant milk (Appendix Table 1A) are shown in figure 3.1. On the first day of lactation, the ash content of the African elephant colostrums was 0.30 % and changed to 0.17 % after 4 days. This is due to the high content of sodium and potassium (Figure 3.2). The content significantly increased up to approximately 0.46 % around the 12^{th} month of lactation and stabilized thereafter.

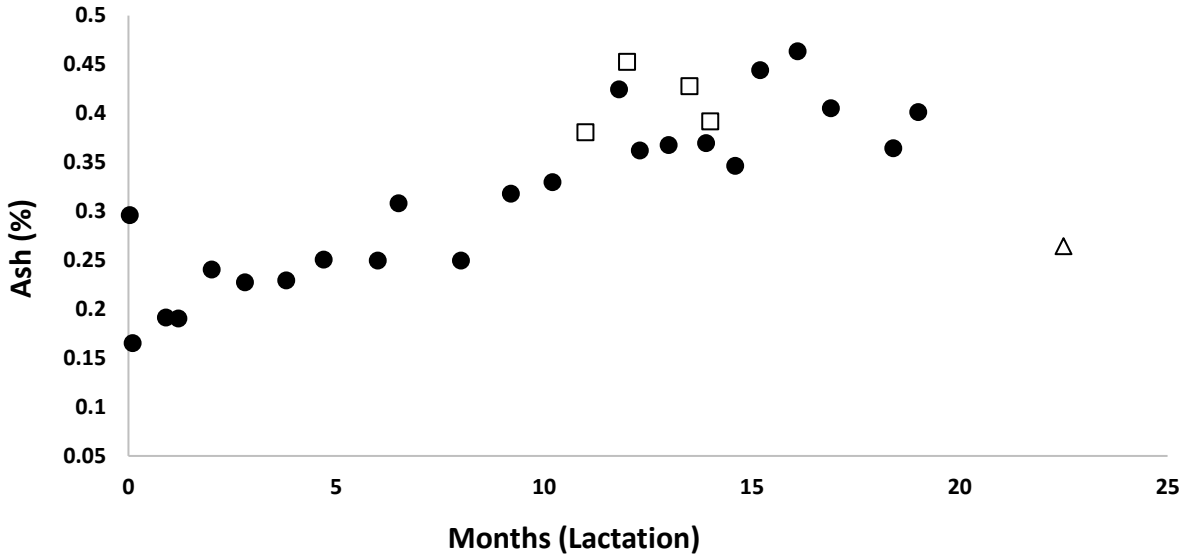

Figure 3.1. Percentage changes in the ash content of three different African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

Figures 3.2 and 3.3. Depict the changes in the mineral composition of African elephant colostrums and mature milk over lactation. The African elephant milk contained high concentrations of K and Na during the first days of lactation, which was 0.120 % and 0.109 % respectively, and decreased to 0.015 % and 0.071 % during the first three days. The content of K increased up to approximately month 11 to 0.186 %, and then fluctuated within a similar concentration and stabilized from the 12th month onwards. The Na content remained constant up to 12 months lactation, followed by fluctuations around 0.025 % thereafter. The Mg showed a similar trend as Na during the lactation period but was lower than the Na concentration throughout lactation.

The content of Ca and P was present in low amounts during the first day of lactation, respectively 0.034 % and 0.019 % as shown in figure 3.3. Ca content showed a sharp increase during the first month of lactation to 0.070 %, and a further increase up to

approximately 12 months lactation to 0.120 %. The concentrations stayed within a similar range thereafter. A similar trend for P content was observed during lactation, increasing to 0.024 % and 0.053 % at the respective times.

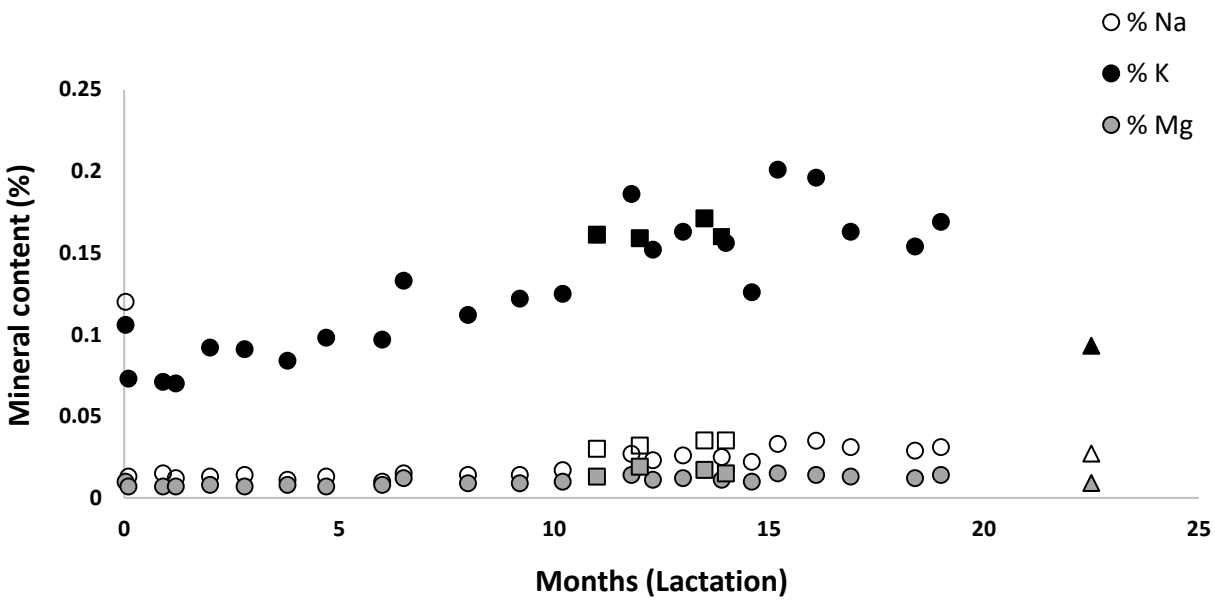

Figure 3.2. Percentage changes in Na, K, and Mg of three different African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

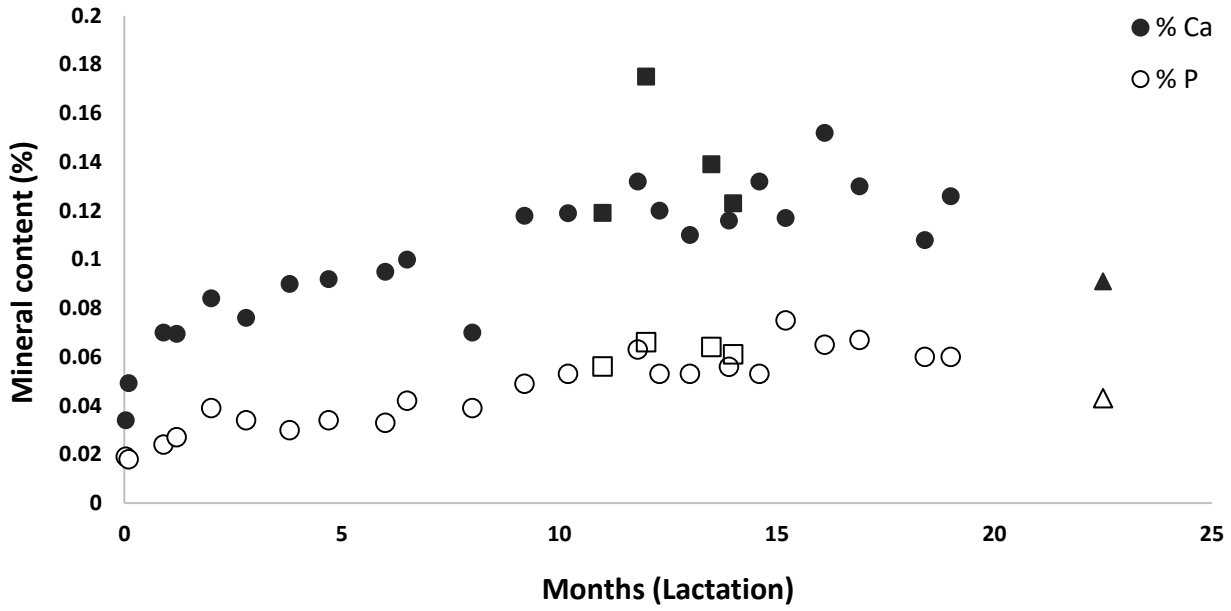

Figure 3.3. Changes in Ca and P of African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

Minor minerals occur in considerably low, yet constant amounts in African elephant milk, with Mg (Figure 3.2), Fe below 0.06 % and Zn, Fe, Cu, Mn, Cd, and Cr in less than 0.001 %. The data obtained from Mussina and Shan, respectively at mid- and late-lactation, is consistent with the trend of ash and mineral changes during lactation within the limits of biological inter-individual variation.

3.2.3. Vitamins

The vitamin content of African elephant colostrums and milk over lactation is shown in Table 3.1. The content of the fat-soluble vitamins A, D and K could not be considered exact because they were below the detection limits of the analytical instrument used. Only vitamins A and E were detected in colostrums at 0.1 and 0.36 mg/kg. The African elephant milk seemed to be void of vitamins during the first month of lactation. Vitamins E and K were detected thereafter at approximately 0.3 mg/kg, and 0.1 µg/kg

respectively. Vitamin A was detected at <0.1 mg/kg milk after 6 months lactation, while traces of vitamin D were detected after 2 months.

Table 3.1. Vitamin content of African elephant milk during lactation.

Months	Vit A (mg/kg)	Vit D (µg/kg)	Vit E (mg/kg)	K (µg/kg)
0.03	0.1	ND	0.36	ND
0.1	ND	ND	0	ND
0.9	ND	ND	0	ND
1.4	ND	ND	< 0.3	< 1.0
2.03	ND	<1.0	< 0.3	< 1.0
2.8	ND	ND	0.33	ND
3.8	ND	ND	0.31	ND
4.7	ND	<1.0	< 0.3	< 1.0
6.03	< 0.1	ND	0.3	< 1.0
7.1	< 0.1	ND	< 0.3	< 1.0
8.1	< 0.1	ND	< 0.3	< 1.0
9.3	< 0.1	ND	< 0.3	< 1.0

3.2.4. Carbohydrates

The dynamic changes in the total carbohydrates of African elephant milk (Appendix Table 2A) during lactation are shown in figure 3.4. The carbohydrate content of African elephant colostrum was 5.0 %, it then increased to 9.2 % after 3 days and eventually to 11.4 % after the first month. After the first month postpartum, a steady decline in total carbohydrate content followed for the rest of the lactation period. The total carbohydrate levels of Mussina and Shan are within the range of the lactation trend.

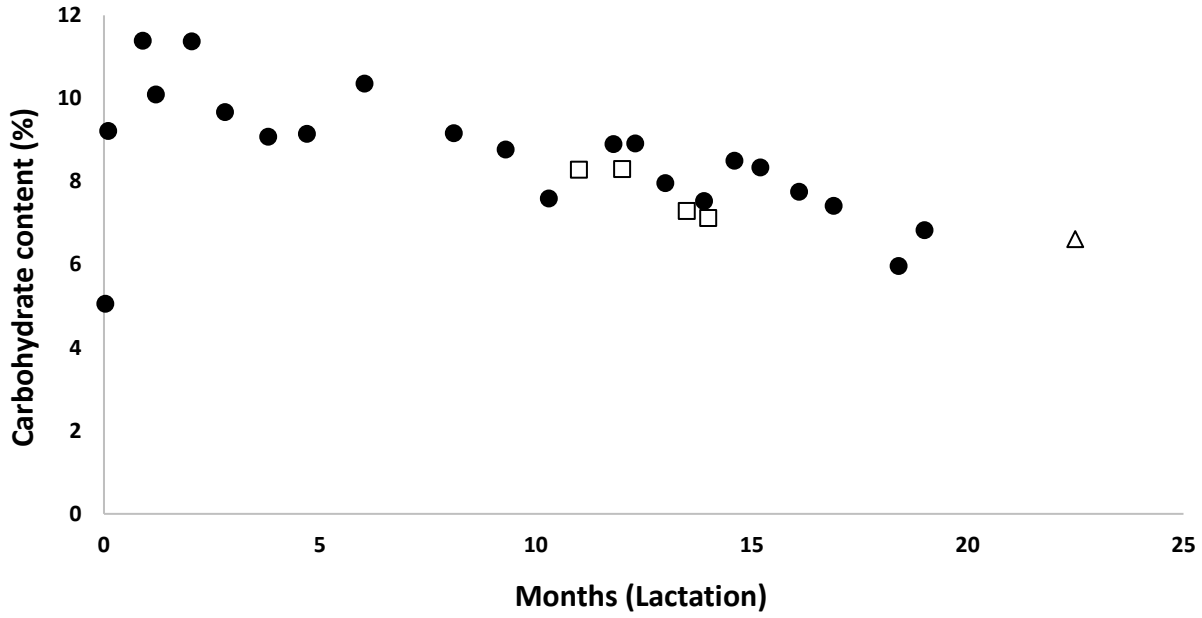

Figure 3.4. Dynamic changes in the total carbohydrates in African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

Changes of the different saccharide fractions during lactation are shown in figures 3.5 and 3.6. The lactose content of African elephant colostrums on the first day was 1.9 % and sharply increased to 5.0 % after 3 days. The lactose content then decreased to approximately 2 % after 9 months, followed by a slow decrease to less than 1.5 % after 19 months.

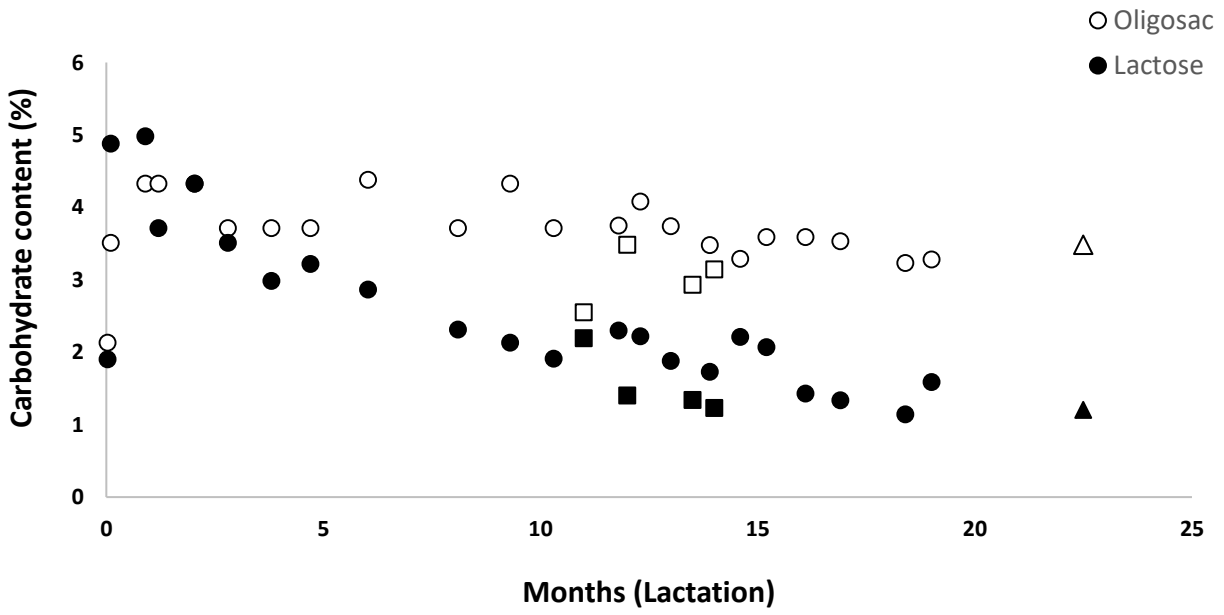

Figure 3.5. Dynamic changes in the saccharide composition in African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

The quantification of the carbohydrate fractions of African elephant milk was based on the lactose content as obtained by HPLC. The concentrations of monosaccharides, lactose and isoglobotriose were determined by HPLC and the phenol-sulphuric acid method for comparison (Table 2A). The results obtained were more or less similar. The concentration of the oligosaccharides was then derived from phenol-sulfuric acid data and re-calculated against the lactose concentration obtained by HPLC. Accurate quantification of acidic oligosaccharides was shown to be best carried out by chemical labelling and separation and quantification of individual oligosaccharides by HPLC (Sumiyoshi *et al.,* 2003). A quantification method other than the phenol-sulphuric acid method of Biogel P2 chromatography peaks has not yet been described. Confidence in the method used here may be provided by the fact that all the milk components of the

milk samples from the African elephants added up to approximately 100 %; the average for all samples was 100.6 ± 9.3 %.

At day zero, the oligosaccharide content of African elephant milk was 2.1 % (Figure 3.5). The content of oligosaccharides increased up to 4.3 % within the first month of lactation and continued within the range of this concentration for the rest of lactation. The quantities of isoglobotriose in the milk of Mussina and Shan, at mid and late lactation respectively, seem to support this trend. African elephant milk oligosaccharides start to become the major sugars from approximately the 4th month of lactation.

The isoglobotriose content of African elephant colostrums was as low as 0.8 % (Figure 3.6). The concentration steadily increased to above 2 % after 2 months lactation and fluctuated at approximately 2.2 % until the 14th month of lactation, after which it seemed to steadily decrease to approximately 1.5 %.

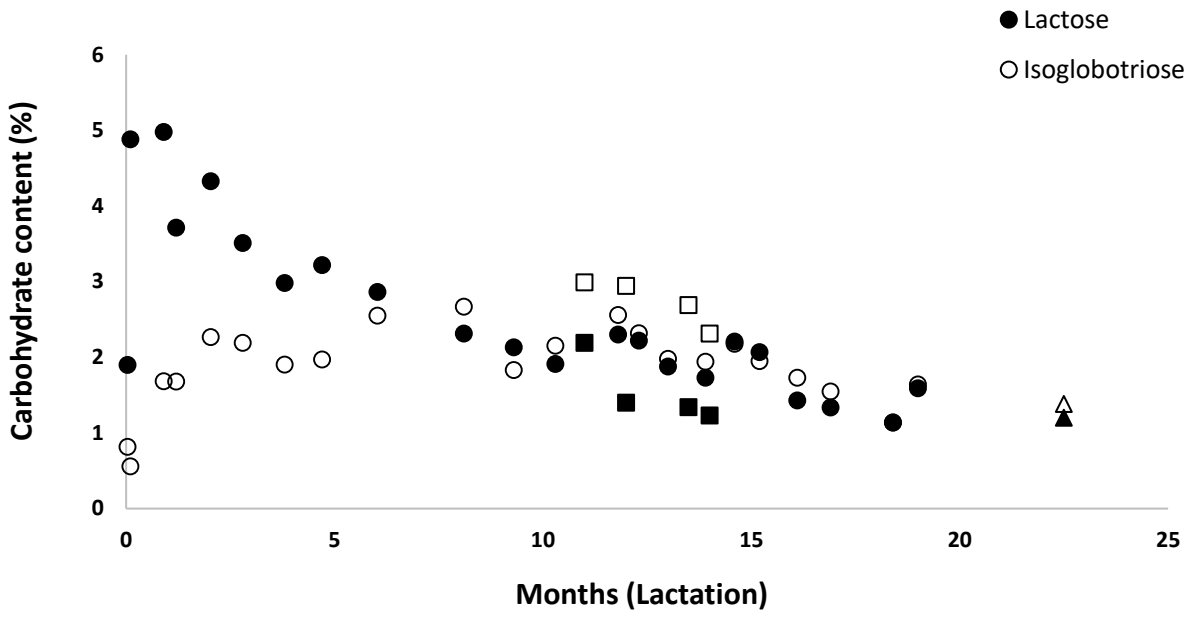

Figure 3.6. Dynamic changes in the saccharide composition in African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

Monosaccharides such as glucose and fucose were present in constant proportions below 0.2 % throughout the lactation period in the African elephant milk (Table 2A).

3.2.5. Proteins

Two different analytical methods were used and subsequently compared in the current study for the analysis of protein in African elephant milk. Two elephant milk samples, months 3.8 and 14.6 of lactation, were subjected to Kjeldahl and Dumas nitrogen combustion analysis in triplicate. The protein content as determined by Kjeldahl and Dumas analyses of a milk sample of 3.8 months lactation was 3.71 ± 0.07% and 3.64 ± 0.19% respectively, and of 14.6-months, 4.68 ± 0.07% and 5.00 ± 0.14% respectively. A paired t-test gave $\alpha = 0.05$ ((NCSS 11 Statistical Software, 2016). The Dumas nitrogen combustion analysis was used to analyze the rest of the elephant milk samples. It has to

be noted that acidic oligosaccharides, which contain N-neuraminic acid, may contribute to the NPN (Takatsu *et al.,* 2017), however, the amounts are almost negligible when the protein content is calculated.

The dynamic change in the protein content of African elephant milk (Appendix Table 2A) during lactation is shown in figure 3.7. The protein content of African elephant colostrums was 4.7 % and decreased to 3.2 % after 1-month lactation. The total protein content in the milk of Mussina and Shan, at mid and late lactation respectively, seem to support the tendency, although a difference in the concentrations of individual elephants due to biological variation was detectable.

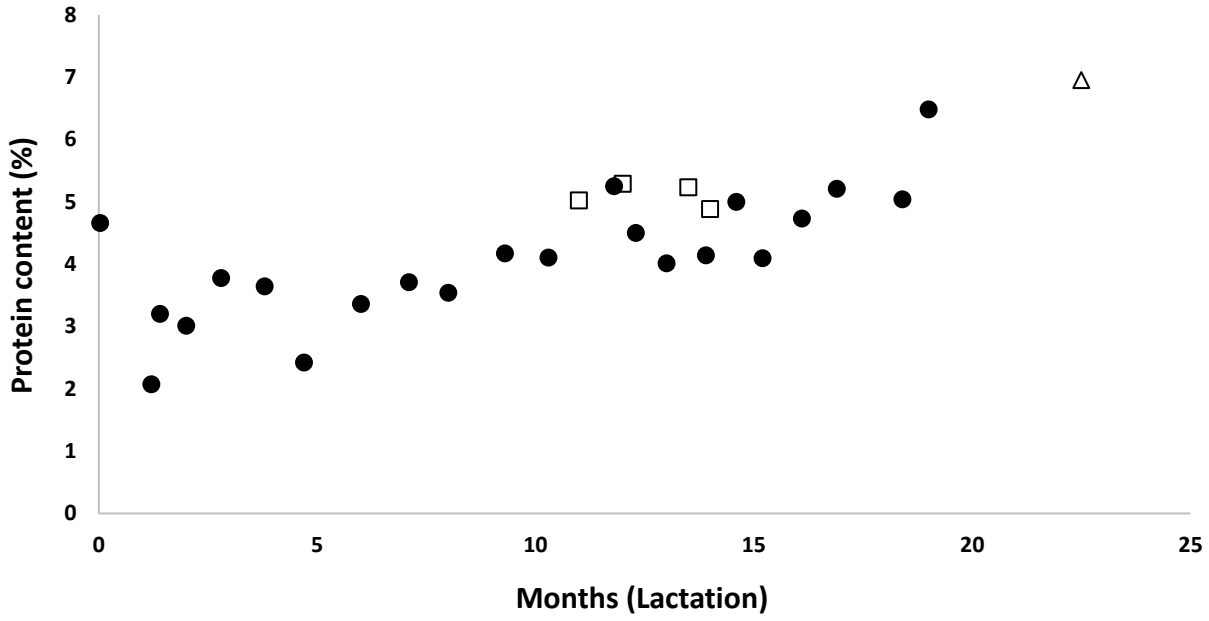

Figure 3.7. Dynamic changes in the total protein content of African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

The variation of the protein fractions during the lactation period is shown in figure 3.8. The casein content in African elephant colostrums was 2.6 %, decreased to approximately 2 % after 5 months, followed by a steady increase with progressing lactation up to approximately 4 % at 19 months postpartum. The whey content in the colostrum was 2 % followed by fluctuations ranging from approximately 0.1 – 2 % throughout the lactation period. The current results suggest that the casein is the dominant protein fraction in African elephant milk throughout the lactation cycle.

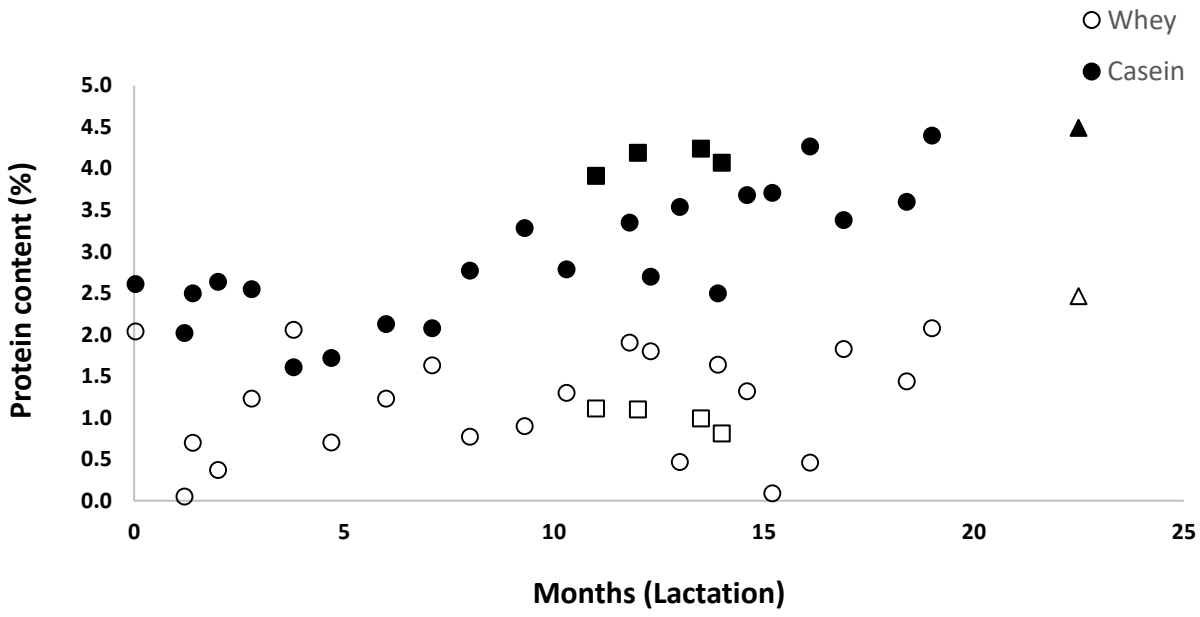

Figure 3.8. Dynamic changes in protein composition of African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

3.2.6. Fat and fatty acids

Two different analytical methods were compared in the current study for the analysis of fat in the African elephant milk. Two elephant milk samples, 3.8- and 14.6-months

lactation, were subjected to the Röse-Gottlieb and a micro-method for fat extraction in triplicate. The fat content as determined by Röse-Gottlieb and a micro-methods of a milk sample of 3.8 months lactation was 2.44 ± 0.20% and 2.63 ± 0.37% respectively, and of 14.6-months, 7.63 ± 0.02% and 7.85 ± 0.46% respectively. A paired t-test gave $\alpha = 0.05$ (NCSS 11 Statistical Software, 2016).. The micro-method was used to analyze the elephant milk samples.

The fat content of the African elephant in colostrums was 2.3 % and stayed at this level for approximately 4 months (Appendix Table 2A and Figure 3.9). After 4 months lactation the content increased steadily to above 12 % after 13 months. Thereafter the fat content seems to fluctuate around 12 %. The total fat content in the milks of Mussina and Shan, at mid and late lactation respectively, seem to support the tendency to stabilize after 13 months lactation, however, at lower amounts of 8 %.

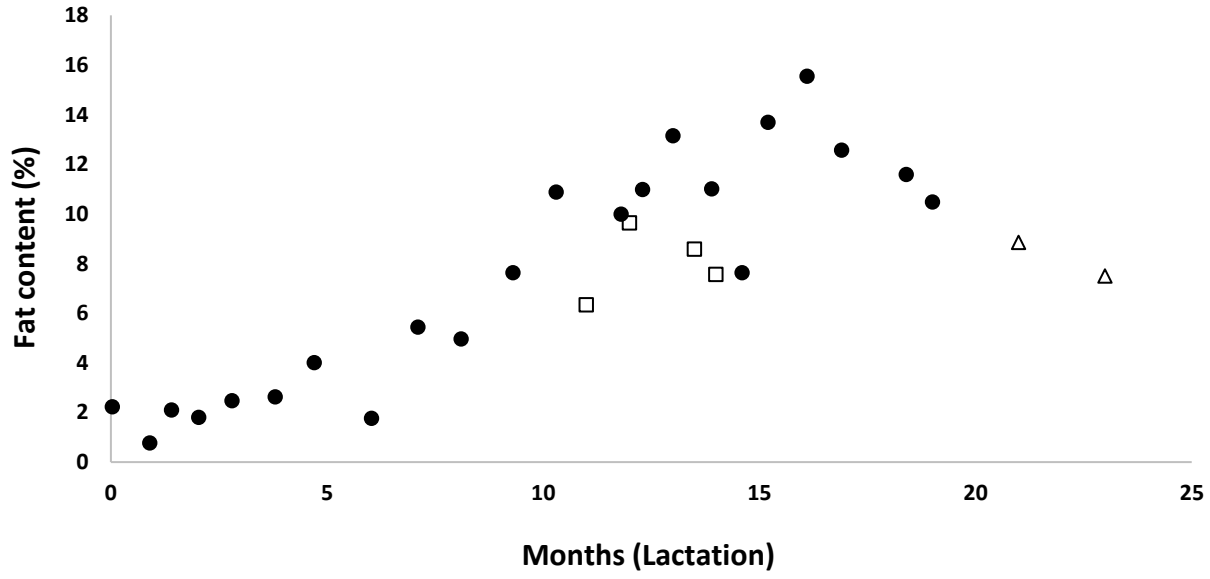

Figure 3.9. Dynamic changes in the total fat in African elephant milk during the lactation period (Bela (circles), Mussina (squares), and Shan (triangles)).

The fatty acid composition of African elephant milk fat displays high amounts of medium-chain fatty acids and low amounts of long-chain and unsaturated fatty acids (Appendix Table 3A). The total content of saturated fatty acids of African elephant milk changes from 72.7 % in colostrums to 94.9 % at 9 months lactation and around 96 % after 19 months of lactation. In general, fatty acids of 10 carbons in length or shorter increases during lactation, while those of 16 carbons and longer decreased in content. Lauric- (12:0) and myristic acid (14:0) content, on the other hand, showed a up-and-down change and then stayed constant. All these changes seem to occur in two phases; drastic changes from day zero to 9 months and slow changes thereafter. The dynamic changes of individual fatty acids in African elephant milk during lactation are depicted in figures 3.10 and 3.11 with selected fatty acids as examples.

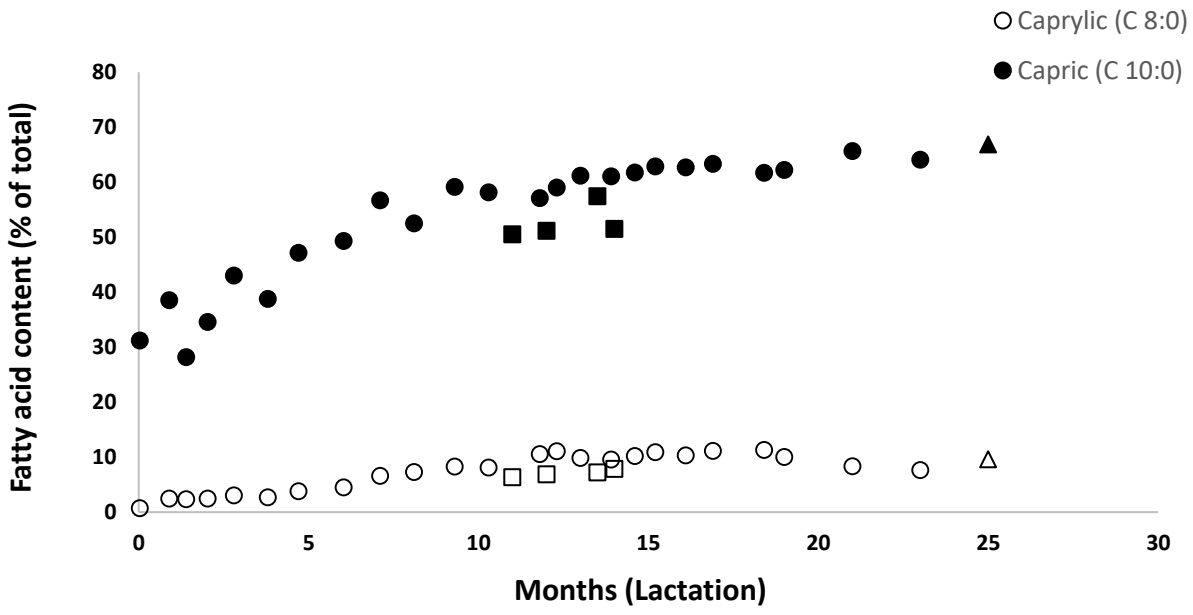

Figure 3.10. Dynamic changes in the medium-chain fatty acids of African elephant milk during lactation (Bela (circles), Mussina (squares), and Shan (triangles)).

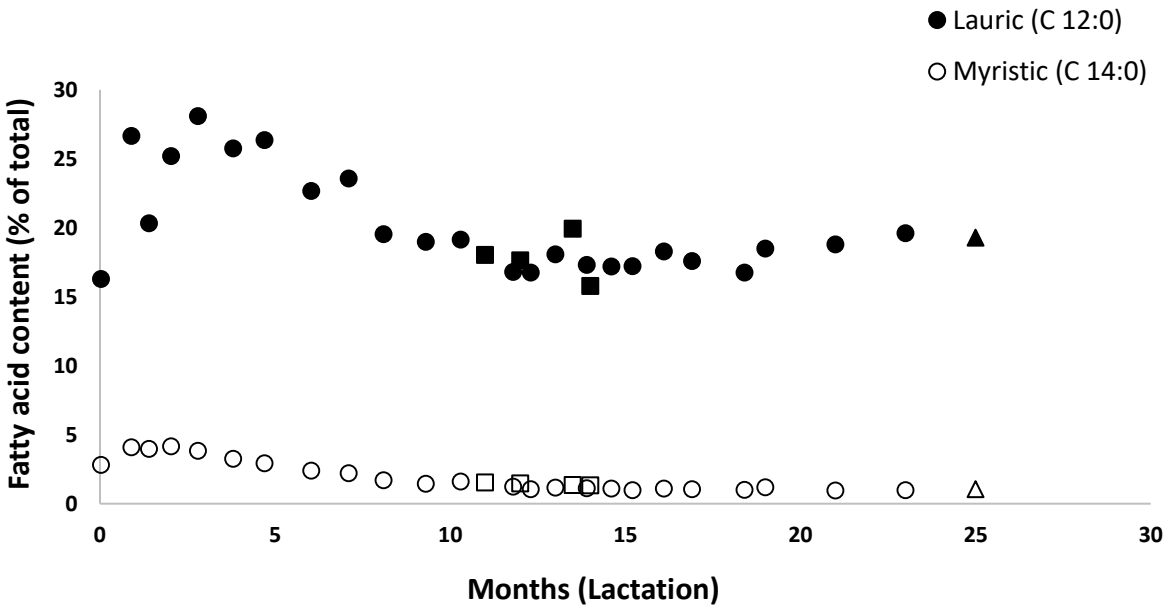

Figure 3.11. Dynamic changes in the medium-chain fatty acids of African elephant milk during lactation (Bela (circles), Mussina (squares), and Shan (triangles)).

Caproic acid (6:0) was found at levels between 0.2 % and 0.4 %. It seemed to occur sporadically before 5 months lactation and consistently thereafter. The presence of short-chain fatty acids in milk is usually associated with ruminant species (Fox *et al.,* 2015a). Therefore, this indicates that there might be some form of fermentation taking place in the gut of the African elephant. The caprylic (8:0) and capric (10:0) acid content of African elephant colostrum was 2.5 % and 31.2 % respectively, changed to 8.3 % and 59.1 % at 9 months lactation and 10.0 % and 62.2 % at 19 months.

The content of lauric (12:0) and myristic (14:0) followed a similar tendency of changes during lactation, at 16.9 % and 2.8 % in colostrums respectively, their content increased to 28.1 % and 3.8 % at 2.8 months, steadily decreased to 18.9 % and 1.4 % at 9 months

and remained constant for the rest of lactation. The content of palmitic (16:0) and oleic (18:1) acids followed a decreasing trend, with 15.2 % and 18.4 % in colostrums respectively, before decreasing to 3.6 % and 4.34 % at 9 months lactation and 1.95 % and 0.1 % at 19 months (Figure 3.12).

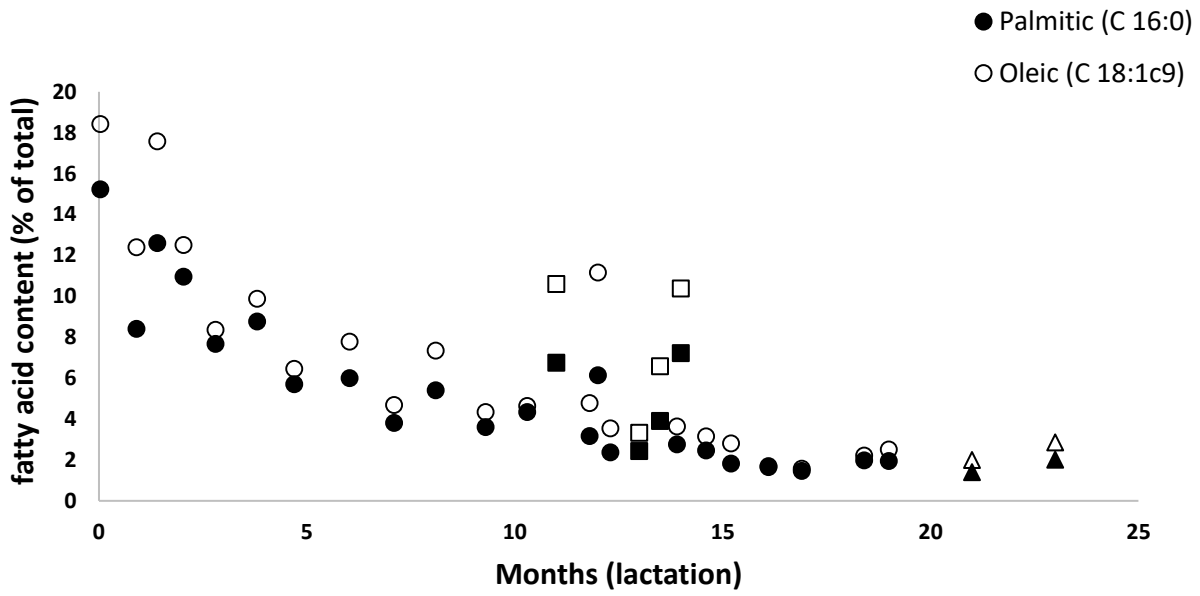

Figure 3.12. Dynamic changes in the long-chain fatty acids of African elephant milk during lactation (Bela (circles), Mussina (squares), and Shan (triangles)).

A low amount of fatty acids of uneven carbon number were observed in small amounts and showed tendencies of change over lactation as the even numbered ones of approximately the same length. The 11:0 occurred at less than 1 % and increased to above 2 %, the 15:0 and 21:0 decreased from 0.2 % to less than 0.01 %, and the 17:0 decreased from 2 % to less than 0.01 %. The same accounted for long-chain unsaturated fatty acids 16:1c9 and 18:3c9,12,15 which decreased from 1 % to less than 0.1 %, while

20:2c11,14, 20:3c11,14,17 and 22:1c13 were detected in trace amount only up to 11 months lactation. No 20:0, 22:0 and 24:0 was detected.

The fatty acid content of milk from Mussina and Shan support the above findings at mid and late lactation respectively. However, milk from Mussina, which was collected from month 11-14, contained higher amounts of unsaturated and lower saturated fatty acids.

3.2.7. Phospholipids

Changes in the fatty acid content of phospholipids of African elephant milk during lactation is shown in Appendix Table 4A. A great variation in the fatty acid composition of total phospholipids was observed over lactation with no tendencies of increase or decrease. As a demonstration, two extreme compositions in subsequent sampling times of Bela, together with the averages over lactation are presented in Table 3.2. Data of elephants Mussina and Shan are also shown.

Table 3.2. Milk total phospholipid fatty acid content of African elephant (Bela) during lactation

		Bela			Mussina	Shan
		sn= 16			sn= 4	sn= 2
Lactation (months)		12.3	13.0			
Fatty acid composition (%):						
Common name:	**Abbreviation:**					
Butyric	C4:0	0.0	0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Caproic	C6:0	0.0	0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 0.00
Caprylic	C8:0	13.3	0.0	1.96 ± 4.49	0.00 ± 3.93	0.00 ± 3.67
Capric	C10:0	54.7	6.9	20.17 ± 14.55	6.30 ± 13.61	6.34 ± 12.99
Hendecanoic	C11:0	1.0	4.5	1.05 ± 1.13	1.56 ± 1.00	1.43 ± 0.99
Lauric	C12:0	7.4	1.5	7.68 ± 4.60	1.93 ± 4.55	5.36 ± 4.4
Tridecoic	C13:0	0.0	0.0	0.03 ± 0.07	0.00 ± 0.06	0.00 ± 0.06

Table 3.2. Continued

		Bela			Mussina	Shan
		Sn= 16			Sn= 4	Sn= 2
Lactation (months)		12.3	13.0			
Fatty acid composition (%):						
Common name:	**Abbreviation:**					
Myristic	C14:0	0.5	1.2	1.85 ± 0.98	1.24 ± 0.88	2.85 ± 0.95
Myristoleic	C14:1c9	0.0	0.0	0.04 ± 0.08	0.51 ± 0.24	0.08 ± 0.24
Pentadecylic	C15:0	0.1	1.7	0.24 ± 0.42	0.00 ± 0.37	0.16 ± 0.35
Pentadecenoic	C15:1c10	0.0	0.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Palmitic	C16:0	3.9	20.3	15.91 ± 6.74	21.46 ± 6.56	23.42 ± 6.72
Palmitoleic	C16:1c9	0.3	1.8	0.81 ± 0.70	0.90 ± 0.68	1.24 ± 0.65
Margaric	C17:0	0.3	0.5	0.55 ± 0.35	0.50 ± 0.31	0.63 ± 0.29
Heptadecenoic	C17:1c10	0.4	0.1	0.41 ± 0.36	0.98 ± 0.42	0.24 ± 0.41
Stearic acid	C18:0	3.6	10.9	13.13 ± 7.20	18.64 ± 7.00	12.74 ± 6.67
Elaidic	C18:1t9	0.1	0.5	0.18 ± 0.24	0.13 ± 0.22	0.08 ± 0.20
Oleic	C18:1c9	11.1	30.0	24.12 ± 6.73	30.50 ± 7.99	30.12 ±8.15
Vaccenic	C18:1c7	0.1	2.7	0.80 ± 0.91	1.19 ± 0.91	1.06 ± 0.89
Nonoadecanoic	C19:0	0.0	0.0	0.50 ± 0.89	0.17 ± 0.78	1.71 ± 0.90
Linolelaidic	C18:2t9,12 (n-6)	0.0	0.6	0.29 ± 0.41	1.30 ± 0.63	0.00 ± 0.62
Linoleic	C18:2c9,12 (n-6)	1.0	9.0	2.89 ± 2.19	3.19 ± 2.69	6.37 ± 2.66
Arachidic	C20:0	0.1	0.0	0.26 ± 0.17	0.19 ± 0.91	0.19 ± 0.14
g-Linolenic	C18:3c6,9,12 (n-6)	0.0	0.4	0.18 ± 0.32	1.35 ± 0.63	0.32 ± 0.61
Eicosenoic	C20:1c11	0.0	0.2	0.23 ± 0.35	0.00 ± 0.31	0.06 ± 0.29
α-Linolenic	C18:3c9,12,15 (n-3)	0.8	0.1	0.73 ± 0.58	0.76 ± 0.54	0.69 ± 0.52
Conjugated linoleic acid	C18:2*c*9,*t*11(*n*-6)(CLA)	0.0	0.0	0.03 ± 0.05	0.14 ± 0.07	0.00 ± 0.07
Conjugated linoleic acid	C18:2*t*10,*c*12(*n*-6)(CLA)	0.0	0.3	0.04 ± 0.08	0.91 ± 0.62	0.02 ± 0.59
Heneicosanoic	C21:0	0.0	0.1	0.04 ± 0.04	0.32 ± 0.20	0.00 ± 0.19
Eicosadienoic	C20:2c11,14 (n-6)	0.0	0.4	1.03 ± 3.20	0.50 ± 2.76	0.47 ± 2.56
Behenic	C22:0	0.0	0.1	0.14 ± 0.24	0.16 ± 0.22	0.15 ± 0.21
Eicosatrienoic	C20:3c8,11,14 (n-6)	0.1	0.2	0.30 ± 0.25	0.70 ± 0.41	0.03 ± 0.39
Erucic	C22:1c13	0.1	0.1	0.43 ± 0.81	0.38 ± 0.70	0.27 ± 0.65
Eicosatrienoic	C20:3c11,14,17 (n-3)	0.1	0.0	0.05 ± 0.05	0.31 ± 0.19	0.09 ± 0.18
Arachidonic	C20:4c5,8,11,14 (n-6)	0.3	2.8	0.45 ± 0.64	0.48 ± 0.57	1.84 ± 0.73
Tricosanoic	C23:0	0.5	1.4	1.30 ± 1.80	0.77 ± 1.61	0.42 ± 1.50
Docosadienoic	C22:2c13,16 (n-6)	0.0	0.1	0.06 ± 0.09	0.20 ± 0.16	0.01 ± 0.15
Eicosopentaenoic	C20:5c5,8,11,14,17 (n-3)	0.0	0.6	0.15 ± 0.20	0.09 ± 0.17	0.03 ± 0.16

Table 3.2. Continued

		Bela			Mussina	Shan
		sn= 16			Sn = 4	Sn= 2
Lactation (months)		12.3	13.0			
Fatty acid composition:						
Common name:	**Abbreviation:**					
Lignoceric	C24:0	0.0	0.0	0.07 ± 0.08	0.22 ± 0.09	0.09 ± 0.09
Nervonic	C24:1c15	0.1	0.2	0.44 ±1.05	0.58 ± 0.91	1.05 ± 0.90
Docosapentaenoic	C22:5c7,10,13,16,19 (n-3)	0.01	0.92	0.33 ± 0.35	1.39 ± 0.64	0.41 ± 0.62
Docosahexanoic	C22:6c4,7,10,13,16,19 (n-3)	0.0	0.0	0.05 ± 0.07	0.05 ± 0.06	0.06 ± 0.06
Fatty acid ratios:						
SFA (%)		85.4	49.0	65.97 ± 9.10	53.45 ± 15.14	55.47 ± 16.63
MUFA (%)		12.3	35.7	27.45 ± 7.56	35.18 ± 8.86	34.20 ± 9.26
PUFA (%)		2.3	15.3	6.58 ± 3.85	11.37 ± 3.93	10.33 ± 4.00
n-6 (%)		1.4	14.2	5.36 ± 4.05	8.67 ± 3.84	9.08 ± 3.78
n-3 (%)		0.92	1.63	1.30 ± 0.72	2.60 ± 0.97	1.28 ± 0.97

With regards to the e phospholipids, the total saturated fatty acids in the phospholipids were low compared to the triacylglycerides, 50-73 % vs 72-96 %. The short-chain fatty acids (4:0 and 6:0) were absent. Of the of medium-chain fatty acids, 8:0 was present only in four milk samples of Bela, between 0.3 % and 33 %, while 10:0 and 12:0 respectively occurred at 10-35 %, and 1-4 %. The 16:0 and 18:1c9 occurred at 3-28 % and 13-37 %. The 10:0 and 12:0 occur in much lower amounts, while the 16:0, 18:0 and 18:1c9 in much higher amounts. Fatty acids of uneven carbon number, 11:0, 15:0, 17:0 and 21:0, occurred at the same low amounts as in the triglycerides, and traces of 19:0 and 23:0 were also detected. However, other than in the triglycerides, mono- and polyunsaturated fatty acids of 16 to 24 carbons in length were detected at amounts below 1 %, and throughout lactation. While the presence and amount of long chain fatty acids were accompanied by low amounts of medium-chain acids, this trend was not obvious in the phospholipids. The fatty acid content of the phospholipids differed throughout lactation, with an up and down variation and with no tendency to change

over lactation. In Table 3.2 the fatty acid composition of phospholipids of two successive milk samples from Bela are shown as examples, together with the averages and standard deviation for all three elephants. The fatty acid composition does not resemble that of the triacylglycerides.

3.2.8. Sterols

Changes in the sterol content of African elephant milk during lactation are shown in Appendix Table 5A. Only the sterol contents after 11 months of lactation are shown. A similar fluctuating trend as observed for phospholipids was observed for the sterol composition, hence, only the average amounts were presented. The content of campesterol, lathosterol, sitosterol, and cholesterol was 8.5 ± 5.9, 5.4 ± 1.0, 7.1 ± 1.5µM and 5.9 ± 5.1 mM respectively. For Mussina, the amounts were 5.4 ± 5.0, 4.0 ± 0.2, 5.9 ± 1.5 and 1.7 ± 0.8 respectively.

3.2.9. Energy

Changes in calculated and measured gross energy during lactation are presented in Appendix table 6A and figure 3.13. The energy levels remained constant at around 25 kCal/100g to approximately 9 months lactation and increased thereafter to around 80 kCal/100g. The calculated gross energy follows the same trend, however at double amounts.

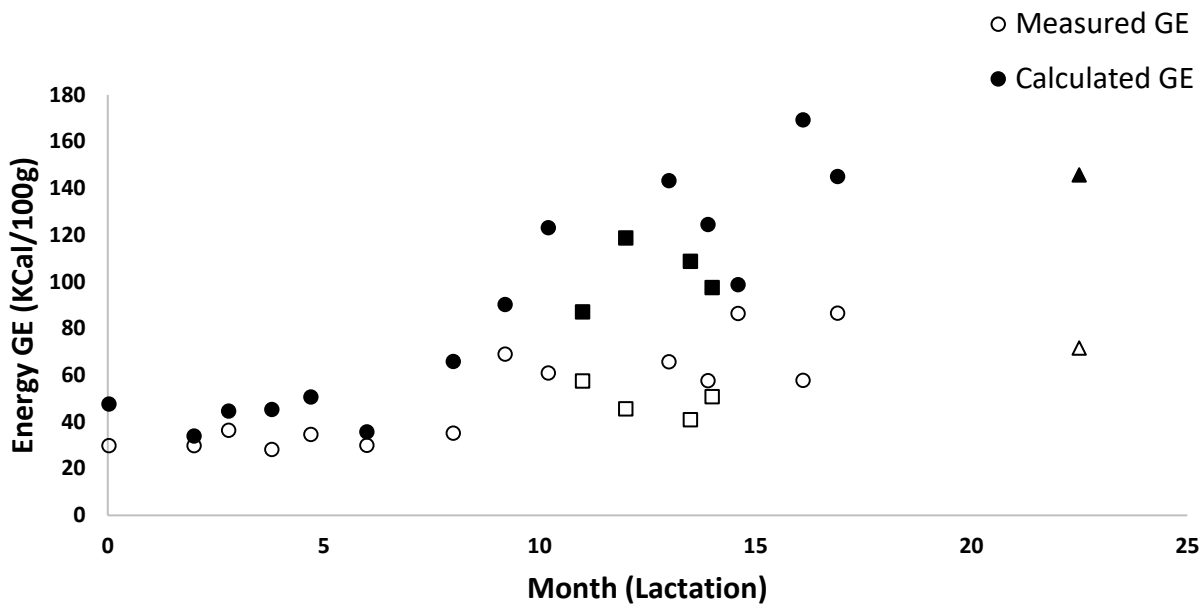

Figure 3.13. Dynamic changes in calculated and measured energy of African elephant milk during lactation (Bela (circles), Mussina (squares), and Shan (triangles)).

Chapter 4

DISCUSSION AND CONCLUSIONS

4.1. Introduction

Milk is the only source of nutrition for a neonate and contains all the components needed for proper growth and development (Hinde *et al.,* 2013)**.** This complex fluid contains different components such as proteins, fat, minerals, carbohydrates and various biofunctional components (Jenness, 1988). As a result, milk has been utilized as a valuable and suitable raw material for the production of milk-related products and is deemed a major component of the human diet. The composition of individual milk components differs between species; the variation may be due to diet, environment, genetic and environmental factors. Studying milk of species other than dairy mammals can help provide insight into many other properties of milk that are not well understood. Moreover, a good understanding of milk may provide knowledge that can help improve the quality of milk and dairy-related products for nutrition or hand-rearing of orphaned infants.

Elephants are one of the survivors of a prehistoric era. The African elephant milk is unique and cannot be compared with the milk of economically exploited dairy animals and other non-dairy species (Osthoff, 2012). African elephant milk has been studied for years and the composition has been described. Certain milk constituents have been described in great detail, and changes of milk components during the lactation period have been pieced together from different elephant species (McCullagh & Widdowson, 1970; Osthoff *et al.,* 2005, 2007a, 2012). The first detailed study on African elephant

milk was conducted by McCullagh and Widdowson (1970), where samples were collected from culled elephants. In the later research Osthoff et al. (2005, 2007a), milk samples were collected from live elephants and the studies provided more insight on the carbohydrates, fat, and protein of the African elephant milk.

4.2. Discussion

In the current study, milk samples were collected from live African elephants. The elephant milk samples were from three different elephant cows. The first elephant (Bella) was sampled from after parturition on day 1 up to 19 months. Milk samples from two additional elephants Mussina and Shan provided milk from 11 to 14 months and 21 to 23 months of lactation respectively. The milk was analyzed and the data showed that the stage of lactation is the greatest cause of variation in milk components of the African elephant. The nutritional parameters of the African elephant milk are unique compared to almost all the mammals that have been studied to date.

The density of the African elephant milk did not change over lactation. The average density was 1.01 ± 0.03, which is lower than that of bovine, goat and sheep milk (Sherbon, 1988; Park *et al.,* 2007). The ash content of African elephant colostrum was present in low amounts, 0.3 %, but steadily increased with advancing lactation to approximately 0.5 % after 12 months. This is different from other species such as human and mare, whose ash levels decrease with the duration of lactation (Vaughan & Kemberling, 1979; Schryver *et al.,* 1986; Summer *et al.,* 2004). However, the data relating to the ash content obtained in the current research is lower than that previously reported on Asian and African elephants (Osthoff, 2012). The ash content follows the content of the major minerals, specifically Na and K, which occur as ions, and P and Ca,

which are mainly associated with the caseins. The mineral composition of African elephant milk varies significantly throughout the lactation period. The major minerals (Na, Ca, K, Mg, and P) increase with progressing lactation. Based on the current data, African elephant and cow milk K (0.159 %) levels seem to be within the same range, while the Na (0.042 %) content occurs in higher amounts in cow milk (Auldist *et al.,* 1998).

The increase in the mineral salts may compensate for the decline in lactose in elephant milk since lactose is the most osmotically active component that regulates the water content in milk. The Ca and P occurred at low amounts in colostrums, respectively at 0.034 % and 0.019 %, and then increased to 0,120 % and 0.053 %. This was lower than the Ca and P content recorded for Asian elephants (Abbondanza *et al.,* 2013) whose Ca content increased from approximately 0.1 % at 2 months to 0.22 % at 28 months, and the P content increased from 0.06 % to 0.12 % at the same stages. The significant increase of Ca and P throughout lactation follows the increase in casein content. Moreover, similar trends were observed in buffalo milk (Yadav & Singh, 1970), where the Ca/P ratio increased over lactation, while the opposite was observed in horse milk (Schryver *et al.,* 1986; Summer *et al.,* 2004). Therefore, the current data and the observations of Abbondanza et al. (2013) appear to conclude that the elephant calf has considerable mineral requirements as it doubles in size.

Vitamins in African elephant milk are present in minor concentrations. The presence, but not the exact content of vitamin A, D, and K could be estimated because the concentrations of the aforementioned vitamins are below the analytical limits of the techniques used. The contents were <0.1 mg/kg for vitamin A, <0.3 mg/kg for vitamin E, <1 µg/kg for vitamins D and K. Vitamin E is the only vitamin component present

throughout the lactation period at 0.36 mg/kg or less. A similar lactation pattern was observed in Asian elephants, where vitamin E was present throughout the duration of lactation (Mainka *et al.,* 1994). The changes in the fat content of African elephant milk over lactation appear to increase the overall fat-soluble vitamin content in elephant milk.

A number of changes in the total saccharides in milk over the lactation period were observed in elephant milk. The total sugars were present in high amounts, 5 % in the colostrums, increased up to the first month of lactation to 11.4 % and steadily decreased for the rest of the lactation to around 6 %. The total sugar levels of other elephants (Mussina and Shan) fell within the same range, which confirmed the lactation pattern. A similar pattern was also reported for Asian elephants, where the total sugar levels dropped over lactation (Abbondanza *et al.,* 2013), however, at half the amounts. Different analytical methods were employed, so that only the trend can be compared, but not the saccharide amounts. The principal sugar in the milk of most mammals is lactose, while the milk of some mammalian species contains a variety of oligosaccharides (Newburg & Neubauer, 1995). The carbohydrate composition of African elephant consists of both lactose and oligosaccharides in high amounts. Lactose was the dominant sugar in the early stages of lactation of the African elephant under study increased from 1.9 % in colostrums to 5.0 % after three days, to constantly decrease to approximately 2 %. Similar observations were made in earlier reports by McCullagh and Widdowson (1970) as well as Osthoff et al. (2005, 2007a). Moreover, the lactose content of reindeer, moose, and black-tailed deer milk was also reported to decrease during the lactation cycle (Mueller, 1977; White & Luick, 1984; Gjøstein *et al.,* 2004). When the changes in lactose content over lactation is viewed in context with the Na and K content described above, it seems as if the increase in osmotic activity is

initially caused by salts, and later by lactose. Lactose is known to regulate the movement of water in milk, which in turn controls the milk volume. Therefore, this is the reason why the milk of eutherian mammals has a dilute nutrient density (Shennan & Peaker, 2000).

The oligosaccharides in African elephant milk increased shortly after parturition from 2.1 % to stabilize at approximately 4.3 %. This is in agreement with earlier reports around a similar lactation time (Osthoff *et al.,* 2005, 2007a). High levels of oligosaccharides are usually linked with marsupials, monotremes, bears, panda bears, proboscides (Asian elephant), sable antelope and gemsbok (Amano *et al.,* 1985; Green & Merchant, 1988; Uemura *et al.,* 2006; Osthoff *et al.,* 2007b).

Isoglobotriose is one of the dominant saccharides in African elephant milk. The concentrations showed a tendency to increase with progressing lactation, the increase was from 0.8 % in colostrums to 2.2 % at 12 months lactation and subsequently decreased to 1.5 % at 19 months and later. It becomes the major saccharide from approximately the 4th month of lactation. This saccharide was also observed in other mammalian species such as the Asian elephant, polar bear, the coati, and the giant panda (Kunz *et al.,* 1999; Urashima *et al.,* 1999a, 2000; Nakamura *et al.,* 2003; Uemura *et al.,* 2006). A similar trend of change was also observed in marsupial milk, where oligosaccharides become main sugars over lactation (Green & Merchant, 1988). In human milk, the complete opposite was observed. There is a significant increase of lactose and a concomitant decrease of oligosaccharides with advancing lactation (Coppa *et al.,* 1993). The presence of high oligosaccharide levels in African elephant milk may be due to different types of galactosyltransferases (Urashima *et al.,* 2009). It has not yet

been established how these transferases are regulated to allow simultaneous synthesis of high amounts of lactose, isoglobotriose and oligosaccharides.

Two different analytical methods were compared in the current study for the analysis of protein in African elephant milk (Kjeldahl & Dumas nitrogen combustion analysis). The Dumas nitrogen combustion analysis was used to analyze the rest of the elephant milk samples. It has to be noted that acidic oligosaccharides, which contain N-neuraminic acid, may contribute to the NPN (Takatsu *et al.,* 2017), however, the amounts are almost negligible when the protein content is calculated. The total protein was present in high concentrations in colostrums at 4.7 %, and decreased to 3.2 % after 1 month, similar to that reported by Osthoff et al. (2005) and steadily increased afterwards for the rest of the lactation period to around 6 %. The total protein content in the milks of Mussina and Shan, at mid and late lactation respectively, seem to support the tendency, although a difference in the concentrations of individual elephants due to biological variation was detectable, however lower than the values reported by (Osthoff *et al.,* 2007a). The trend in the protein levels over lactation is consistent with the previous work of McCullagh and Widdowson (1970), Osthoff et al. (2005,2007a) and Abbondanza et al. (2013). The caseins were the predominant protein in the African elephant milk. The casein content increased from 2.6 % to 4 % and the whey steadily decreased from 2 % to 1 % with advancing lactation. However, this is different from other monogastric species such as pig, human, cheetah, and African lion, which have high concentrations of whey proteins, especially during early lactation (Csapó *et al.,* 1996; De Waal *et al.,* 2004; Osthoff *et al.,* 2006).

The fat content of the African elephant milk increased with advancing lactation from 2.3 % in colostrums to above 12 %. The total fat content in the milks of Mussina and Shan,

at mid and late lactation respectively, seem to support the tendency to stabilize around mid-lactation, however, at lower amounts. In a previous report, the fat content of milk from a number of individual elephants seemed to stabilize at approximately 15 % (Osthoff, 2012). It therefore, seems as if the fat content is affected by biological variation. Nevertheless, the increase in fat content with advancing lactation appears to be a characteristic of the African elephant (McCullagh & Widdowson, 1970; Osthoff *et al.,* 2005, 2007a). Moreover, the increase in milk fat appears to compensate for the decrease in carbohydrates with regards to energy provision for the elephant calf. Similar observations were made on Asian elephants with an increase from 7.5 % to 15 % and 17.5 % fat (Abbondanza *et al.,* 2012).

The fatty acid profile of African elephant is characterized by high concentrations of medium-chain saturated fatty acids. This is different from most mammals whose fatty acid composition consist mostly of long-chain and unsaturated fatty acids. The total content of saturated fatty acids of African elephant milk changes from 72.7 % in colostrums to 94.9 % at 9 months lactation and around 96 % after 19 months of lactation. In general, the fatty acids of 10 carbons in length and shorter, increase during lactation, while those of 14 carbons and longer decrease, with lauric acid (12:0) expressing little change. Caproic acid (6:0) was found at levels between 0.2 % and 0.4 %. It seemed to occur sporadically before 5 months lactation and consistently thereafter. The presence of short-chain fatty acids in milk is usually associated with ruminant species (Fox *et al.,* 2015a). Therefore, this indicates that there might be some form of fermentation taking place in the gut of the African elephant. All these changes seem to occur in two phases; drastic changes from day zero to 9 months and slow changes thereafter. Although the phases of change were noted in a previous report (Osthoff, 2012), a lack of data in the early to mid-lactation range made an accurate positioning

impossible. It is to be noted that the pattern of changes in milk fatty acids observed here, differs from that reported by McCullagh & Widdowson (1970). They reported amounts of 50 % 10:0 at 3 months, while the current results and earlier results of Osthoff (2012) show that, in spite of large biological variation, this level is only reached after at least 7 months of lactation. In the current work, the stage of lactation is according to the real age of the elephant calf, while McCullagh and Widdowson based the ages of the culled calves on dentition, the stage of dental development in the study. This seems to have caused an underestimation of the stage of lactation and therefore an inaccurate description of the changes of milk fatty acids over lactation.

The amounts of caprylic acid in African elephant milk are significantly higher than that of the cow, mare, and human (Jensen *et al.,* 1990; Csapó *et al.,* 1995). The only species that display high concentrations of short/medium chain fatty acids known thus far, are the rabbit (8:0 – 10:0), and both Indian and white rhinoceros (10:0 - 12:0) (Hall, 1971; Klös *et al.,* 1974; Demarne *et al.,* 1978, Osthoff *et al.,* 2008). The described tendency of changes towards shorter milk fatty acids over lactation has not been documented for any other mammal. The only reason for such a change may lie in the energy provision of the fat. Comparison of the energetic balance of the total oxidation of the fatty acids between milk fat from cow and elephant shows that the elephant may provide approximately 6 % less energy at late lactation. This is compensated by the synthesis of medium length fatty acids providing a saving of energy to the elephant cow (Voet & Voet, 2011). The energy density of elephant milk is therefore increased by the fat content (Abbondanza *et al.,* 2012), and not by the change in fatty acid composition.

The African elephant milk phospholipids of medium-chain fatty acids, such as capric and lauric acid, are present in low concentrations compared to the triacylglycerides, while

the long-chain fatty acids such as palmitic, stearic, and oleic acid were present in high levels. Long-chain unsaturated fatty acids were also present in the phospholipids, but not in the triacylglycerides. In this sense, the fatty acid composition, therefore, does not resemble that of the triacylglycerides, but is rather comparable with bovine, pig and human phospholipids by having high amounts of long-chain fatty acids (Bitman *et al.,* 1984, 1990; Wood *et al.,* 2008; Singh & Gallier, 2017).

The content of cholesterol of African elephant milk at approximately 5.9 mM and the concentration is higher than that of cow and human milk (Clark *et al.,* 1982; Robert G. Jensen, 2002a). Cholesterol occurs in the milk fat globule membrane and is carried over from the cell membrane of the milk-secreting cell (Ontsouka & Albrecht, 2014). The campesterol, lathosterol, and sitosterol occurred at approximately 8.7 µM. These originate from the plant food ingested by the elephant cow and is transferred to the milk from the blood. The amounts are also present in small amounts in cow milk (MacGibbon & Taylor, 2009). The cholesterol content fluctuated up and down over lactation, without a specific pattern, but may follow the fluctuation of the fatty acids of the phospholipids.

The up and down fluctuation of the fatty acid composition of the phospholipids, as well as the cholesterol, needs discussion. The fatty acids of the phospholipids in the milk of cow (Bitman & Wood, 1990) and humans (Lauber & Reinhardt, 1979; Bitman *et al.,* 1984) seem not to change over lactation, as very small variations in composition were reported, derived from the low standard deviations. In these mammals, milk is synthesized and secreted constantly, and the milk stored in a cistern. Parts of the milk cell membrane are therefore removed at a constant rate as the fat droplets are enveloped. The cell membrane is then regenerated at a constant rate. This is important

because the flexibility of the membrane has to be maintained. In the African elephant, milk synthesis and secretion seems to not be at a constant rate, as it is stimulated on demand by suckling. The fluctuation in fatty acid content in the milk phospholipids of elephant suggests that the milk secretion might deprive the cell wall of its long-chain fatty acids, specifically the unsaturated ones. When suckling is stopped and then followed by a resting time, the membrane is restored.

It seems as if restoration may take some time. If suckling events follow in short succession, the phospholipids are replaced from the phospholipids within the cell lumen that contain high amounts of medium-chain fatty acids. The exchange with medium-chain fatty acids should not affect the flexibility of the cell membrane, based on their low melting temperature as triacylglycerides (Osthoff *et al.,* 2011). This may also explain the fluctuation of the cholesterol because the cholesterol in milk originates from the milk synthesis cell membrane, and slow restoration may deprive the membrane. This explanation is based on the only possible reason in that the suckling history before milk collection is not known. More research would be required as proof.

The energy levels do not vary much in the early months of lactation. An increase is observed from the 10^{th} month of lactation onwards, from 20 kCal/100g to 80 kCal/100g. This is due to the increase in the milk nutrients (protein, fat, and saccharides). A similar observation of the increase in energy was made in the study of Asian elephants, however, on calculated energy values (Abbondanza et al, 2013). These researchers commented that the calculated energy values were most probably an over-estimation. The calculated energy of African elephant is double that of the experimentally measured energy. This great deviation may be due to the nutrients of African elephant milk that are not of the same composition as that described by Perrin (1958), who devised the

calculation formula. Perrin had to make separate mathematical compensations in order to obtain calculated values that were similar to the experimental calorific values. Moreover, the nutrient content of the milks of species included by Perrin (1958) did not differ as much from each other. At the early months of lactation, elephant milk did not differ too much from most of the mammals in the publication with regard to the content of fat, protein, and saccharides. Hence the difference in the calculated and experimentally measured values did not differ too much. However, after 10 months lactation, the calculated values are twice as high as experimental ones, which is due to the high content of all nutrients on the one hand, as well as the difference in the detailed composition of each of the nutrients.

Furthermore, the fatty acid and carbohydrate composition of African elephant milk differ substantially from other mammalian species. Perrin (1958) based the calculations on species of which the milk composition consists of fat that is very similar to cow's milk, and contain long-chain fatty acids. That means that there is more fatty acid per gram fat than glycerol, while in elephant milk, due to the high amount of medium-chain fatty acids, there is more glycerol per gram fat; approximately 1.2 times. It is possible that this may lead to an over-estimation of the energy from fat, however, this has to be determined experimentally. The greatest contributor to the high calculated energy values may be the carbohydrates. Perrin's (1958) milk carbohydrates were all assumed to be lactose, while in elephant milk lactose is only one-third of the total carbohydrates, and the energy value of isoglobotriose and oligosaccharides is not known. Therefore, this concludes that the formula used to calculate the values of energy only applies to milk similar to cow or other ruminants and adjustments need to made for the inclusion of other mammalian species especially the non-dairy ones (Perrin, 1958; Petzinger *et al.,* 2014).

4.3. Conclusions

The research presented here added to existing data on the dynamic changes observed in the nutrient composition of African elephant milk over lactation. The sampling of milk over short intervals filled in gaps and milk of two additional elephants added to the confirmation of the unique changes only observed in these species, as well as to indicate the extent of biological variation.

The information on minerals, vitamins, separate saccharide fractions, phospholipids, and energy add new information. The information on the phospholipids and sterols may provide new insight into the biochemistry of the milk fat globule membrane and the milk-secreting cell, although more in-depth research is required.

The data of all parameters combined show that the lactation of the African elephant may be divided into three stages: colostrums, which does not last longer than two or three days, a 12-month period of constant change, and mature milk from approximately twelve months until the end of lactation.

4.4. Future research

Since the nutritional parameters of African elephant milk continuously change over the lactation cycle, future experimental work could be directed towards the kinetics and biochemical regulation of the nutrients, specifically those that show drastic changes over lactation. These include the fatty acid synthesis, specifically the fatty acid synthase. It would also be of interest to study the individual neutral and acidic oligosaccharides

over lactation, to find out whether they exchange each other, although the total amount stays constant. Also, how the galactosyltransferases are regulated to allow simultaneous high concentrations of lactose, isoglobotriose and oligosaccharides. An in-depth investigation of the fluctuation of the fatty acids of phospholipids, and fluctuation of cholesterol content would improve the understanding of the milk fat globule membrane and milk cell membrane; not only in the African elephant but in all species in general.

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Appendix

Table 1A. Shows the changes in the mineral content of African elephant milk during the lactation period.

Months	% Ca	% P	% Na	% K	% Mg	% Zn	% Fe	% Cu	% Mn	% Cd	% Cr	Ash
0.03	0.034	0.019	0.120	0.106	0.010	0.0008	0.0034	0.0001	0.0001	0.0003	0.0023	0.30
0.1	0.049	0.018	0.013	0.073	0.007	0.0005	0.0027	0.0001	0.0001	0.0003	0.0012	0.17
0.9	0.070	0.024	0.015	0.071	0.007	0.0005	0.0024	0.0001	0.0001	0.0004	0.0010	0.19
1.2	0.070	0.027	0.012	0.070	0.007	0.0004	0.0025	0.0001	0.0001	0.0005	0.0012	0.19
2	0.084	0.039	0.013	0.092	0.008	0.0007	0.0021	0.0001	0.0001	0.0006	0.0010	0.24
2.8	0.076	0.034	0.014	0.091	0.007	0.0006	0.0029	0.0001	0.0001	0.0007	0.0011	0.23
3.8	0.090	0.030	0.011	0.084	0.008	0.0007	0.0035	0.0002	0.0001	0.0008	0.0010	0.23
4.7	0.092	0.034	0.013	0.098	0.007	0.0007	0.0037	0.0002	0.0001	0.0009	0.0011	0.25
6	0.095	0.033	0.01	0.097	0.008	0.0006	0.0038	0.0002	0.0001	0.0010	0.0009	0.25
6.5	0.100	0.042	0.015	0.133	0.012	0.0008	0.0030	0.0001	0.0001	0.0010	0.0012	0.31
8	0.070	0.039	0.014	0.112	0.009	0.0004	0.0026	0.0001	0.0001	0.0012	0.0012	0.25
9.2	0.118	0.049	0.014	0.122	0.009	0.0006	0.0025	0.0001	0.0001	0.0013	0.0013	0.32
10.2	0.119	0.053	0.017	0.125	0.010	0.0007	0.0021	0.0001	0.0001	0.0013	0.0014	0.33
11.8	0.132	0.063	0.027	0.186	0.014	0.0005	0.0015	0.0002	0.0001	0.0000	0.0002	0.42
12.3	0.120	0.053	0.023	0.152	0.011	0.0005	0.0019	0.0002	0.0001	0.0000	0.0003	0.36
13	0.110	0.053	0.026	0.163	0.012	0.0006	0.0023	0.0003	0.0001	0.0000	0.0003	0.37
13.9	0.116	0.056	0.025	0.160	0.011	0.0005	0.0010	0.0001	0.0001	0.0000	0.0001	0.37
14.6	0.132	0.053	0.022	0.126	0.010	0.0005	0.0023	0.0002	0.0001	0.0000	0.0002	0.35
15.2	0.117	0.075	0.033	0.201	0.015	0.0008	0.0019	0.0002	0.0001	0.0000	0.0002	0.44
16.1	0.152	0.065	0.035	0.196	0.014	0.0006	0.0005	0.0002	0.0001	0.0000	0.0001	0.46
16.9	0.130	0.067	0.031	0.163	0.013	0.0006	0.0002	0.0002	0.0001	0.0000	0.0001	0.41
18.4	0.108	0.060	0.029	0.154	0.012	0.0006	0.0005	0.0003	0.0001	0.0000	0.0001	0.36
19	0.126	0.060	0.031	0.169	0.014	0.0006	0.0004	0.0002	0.0001	0.0000	0.0001	0.41
22.5	0.091	0.043	0.027	0.093	0.009	0.0004	0.0003	0.0002	0.0001	0.0000	0.0001	0.26

Table 1B. Shows the changes in the mineral content of milk of elephant two (Mussina) and three (Shan) at mid- and late-lactation.

Months	% Ca	% P	% Na	% K	% Mg	% Zn	% Fe	% Cu	% Mn	% Cd	% Cr	Ash
11	0.119	0.056	0.03	0.161	0.013	0.0006	0.0007	0.0002	0.0001	0.0000	0.0001	0.38
12	0.175	0.066	0.032	0.159	0.019	0.0007	0.0006	0.0002	0.0001	0.0000	0.0001	0.45
13.5	0.139	0.064	0.035	0.171	0.017	0.0007	0.0004	0.0003	0.0001	0.0000	0.0001	0.43
14	0.123	0.061	0.035	0.156	0.015	0.0007	0.0006	0.0003	0.0001	0.0000	0.0001	0.39
22.5	0.091	0.043	0.027	0.093	0.009	0.0004	0.0003	0.0002	0.0001	0.0000	0.0001	0.26

Table 2A. Milk nutrient content of an African elephant (Bela) during lactation

Name of Elephant										**Bela**											
Time of lactation (months)	**0.03**	**0.9**	**1.4**	**2.03**	**2.8**	**3.8**	**4.7**	**6.03**	**7.1**	**8.1**	**9.3**	**10.3**	**11.8**	**12.3**	**13**	**13.9**	**14.6**	**15.2**	**16.1**	**16.9**	**18.4**	**19**
Chemical properties (%)																						
% Density	1.033	1.030	1.030	1.034	1.035	1.042	1.158	1.036	1.277	1.041	1.028	1.021	0.980	0.980	1.023	1.023	0.979	1.020	1.033	1.016	1.010	1.190
% Moisture	89.7			87.7	86.2	88.1	86.1	89.1		88.3	84.0	81.7			76.8	78.2	63.7		84.7	75.1		
% Fat	2.2	0.8	2.1	1.8	2.5	2.6	4.0	1.8	5.4	5.0	7.6	10.9	10.0	11.0	13.2	11.0	7.6	13.7	15.5	12.6	11.6	10.0
% FFDM	10.6	8.1	8.8	9.2	9.9	9.2	9.3	10.0	8.4	11.1	9.9	11.1	10.0	9.7	9.6	9.7	10.3	9.4	10.0	10.2	11.0	10.0
% Protein	4.7		3.2	3.0	3.8	3.6	2.4	3.4	3.7	3.5	4.2	4.1	5.3	4.5	4.0	4.1	5.0	4.1	4.7	5.2	5.0	6.5
% Whey Protein	2.0		0.7	0.4	1.2	2.1	0.7	1.2	1.6	0.8	0.9	1.3	1.9	1.8	0.5	1.6	1.3	0.1	0.5	1.8	1.4	2.1
% Casein	2.6		2.5	2.6	2.6	1.6	1.7	2.1	2.1	2.8	3.3	2.8	3.4	2.7	3.5	2.5	3.7	3.7	4.3	3.4	3.6	4.4
% NPN	0.1		0.1	0.1	0.0	0.0	0.3	0.1	0.2	0.3	0.2	0.1	0.1	0.12	0.2	0.1	0.1	0.2	0.0	0.1	0.1	0.1
% Lactose	1.9	5.0		4.3	3.5	3.0	3.2	2.9		2.3	2.1	1.9	2.3	2.2	1.9	1.7	2.2	2.1	1.4	1.3	1.1	1.6
% Galactose	0.1	0.1		0.1	0.1	0.1	0.0	0.1		0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.2
% Fucose	0.2	0.3		0.4	0.2	0.4	0.2	0.5		0.4	0.4	0.5	0.2	0.2	0.3	0.3	0.6	0.6	0.9	0.8	0.2	0.2
% Oligosaccharide (HPLC)	2.1	4.3		4.3	3.7	3.7	3.7	4.4		3.7	4.3	3.7	3.8	4.1	3.7	3.5	3.3	3.6	3.6	3.5	3.2	3.3
% Isoglobotriose (HPLC)	0.8	1.7		2.3	2.2	1.9	2.0	2.6		2.7	1.8	2.2	2.7	2.3	2.0	1.9	2.2	2.0	1.7	1.6	1.1	1.6
% Isoglobotriose (phenol met)	1.0	0.5		2.4	2.2	2.1	1.8	2.2		2.6	2.0	1.7	2.3	2.2	1.7	1.9	1.6	1.9	1.7	1.2	1.6	1.8
% Monosaccharide (phenol met)	0.2	0.3		0.4	0.5	0.4	0.5	0.6		0.5	0.5	0.6	0.3	0.3	0.4	0.6	0.9	0.8	0.9	0.9	0.6	0.6

Table 2B. Milk nutrient content of an African elephant during lactation

Name of Elephant		**Mussina**			**Shan**	
Time of lactation (months)	**11**	**12**	**13.5**	**14**	**21**	**22.5**
Chemical properties (%):						
% Density	0.978	0.980	0.975	1.001		0.944
% Moisture	76.5	82.9	83.9	80.2		84.2
% Fat	6.3	9.6	8.6	7.6	8.9	7.5
% FFDM	9.5	8.9	9.8	10.0	10.5	10.6
% Protein	5.0	5.3	5.2	4.9		7.0
% Whey	1.1	1.1	1.0	4.1		4.5
% Casein	3.9	4.2	4.2	0.8		2.5
% NPN	0.1	0.1	0.3	0.2		0.2
% Lactose	2.2	1.4	1.3	1.2		1.2
% Galactose	0.2	0.2	0.1	0.1		0.2
% Fucose	0.4	0.3	0.2	0.3		0.3
% Oligosaccharide	2.6	3.5	2.9	3.1		3.5
% Isoglobotriose	3.0	2.9	2.7	2.3		1.4
% Isoglobotriose (Phenol method	3.2	3.1	2.6	2.5		1.6
% Monosaccharides (Phenol method)	0.5	0.5	0.3	0.5		0.3

Table 3A. Milk Fatty Acid composition of an African elephant (Bela) during lactation

		Bela											
Time of Lactation (months)		**0.03**	**0.9**	**1.4**	**2.03**	**2.8**	**3.8**	**4.7**	**6**	**7.1**	**8.1**	**9.3**	**10.3**
Fatty acid composition (%):													
Common name:	**Abbreviation:**												
Butyric	C4:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Caproic	C6:0	0.0	0.1	0.0	0.0	0.1	0.0	0.1	0.0	0.3	0.2	0.2	0.2
Caprylic	C8:0	2.5	2.9	2.3	2.5	3.0	3.5	3.8	4.5	6.6	7.3	8.2	8.1
Capric	C10:0	31.2	38.5	30.0	34.6	43.0	41.2	47.2	46.5	53.7	52.5	59.1	58.1
Hendecanoic	C11:0	0.2	0.7	0.5	1.8	1.9	2.5	3.1	2.6	2.0	1.4	1.4	1.3
Lauric	C12:0	16.9	26.7	22.4	25.2	28.1	24.5	26.3	23.0	23.6	19.6	19.0	19.2
Tridecoic	C13:0	0.0	0.1	0.1	0.3	0.3	0.4	0.5	0.3	0.2	0.1	0.1	0.1
Myristic	C14:0	2.8	4.1	4.0	4.2	3.8	3.2	2.9	2.4	2.2	1.7	1.5	1.6
Myristoleic	C14:1c9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pentadecylic	C15:0	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.1	0.2
Pentadecenoic	C15:1c10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Palmitic	C16:0	15.2	8.4	12.6	11.0	7.7	8.8	5.7	6.0	3.8	5.4	3.6	4.3
Palmitoleic	C16:1c9	1.6	0.8	1.2	0.7	0.4	0.4	0.3	0.4	0.4	0.3	0.2	0.2
Margaric	C17:0	1.6	0.3	1.7	1.9	0.3	1.4	0.4	2.0	0.2	0.9	0.2	0.2
Heptadecenoic	C17:1c10	0.7	0.0	0.6	0.3	0.0	0.2	0.2	0.2	0.0	0.2	0.1	0.0
Stearic acid	C18:0	1.7	1.3	1.6	1.3	0.9	1.2	0.9	1.2	0.7	0.9	0.6	0.7
Elaidic	C18:1t9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Oleic	C18:1c9	18.4	12.4	17.6	12.5	8.4	9.9	6.5	7.8	4.7	7.3	4.3	4.6
Vaccenic	C18:1c7	3.1	1.4	2.3	1.0	0.7	0.8	0.6	0.8	0.5	0.7	0.4	0.4
Linolelaidic	C18:2t9,12 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Linoleic	C18:2c9,12 (n-6)	2.1	0.8	1.5	1.1	0.7	1.0	0.9	1.1	0.7	0.6	0.3	0.3
Arachidic	C20:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
γ-Linolenic	C18:3c6,9,12 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Eicosenoic	C20:1c11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
α-Linolenic	C18:3c9,12,15 (n-3)	1.0	0.6	0.8	0.6	0.5	0.5	0.3	0.4	0.3	0.6	0.4	0.4

Conjugated linoleic acid (CLA)	C18:2c9t11 (n-6) (CLA)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Heneicosanoic	C21:0	0.4	0.5	0.4	0.6	0.0	0.3	0.0	0.6	0.0	0.1	0.0	0.0
Eicosadienoic	C20:2c11,14 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Behenic	C22:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Eicosatrienoic	C20:3c8,11,14 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Erucic	C22:1c13	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Eicosatrienoic	C20:3c11,14,17 (n-3)	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0
Arachidonic	C20:4c5,8,11,14 (n-6)	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tricosanoic	C23:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Docosadienoic	C22:2c13,16 (n-6)	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Eicosopentaenoic	C20:5c5,8,11,14,17 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lignoceric	C24:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nervonic	C24:1c15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Docosapentaenoic	C22:5c7,10,13,16,19 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Docosahexanoic	C22:6c4,7,10,13,16,19 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fatty acid ratios:													
SFA (%)		72.7	83.8	75.8	83.5	89.2	87.1	91.2	89.3	93.5	90.1	94.1	94.0
MUFA (%)		23.9	14.7	21.7	14.6	9.5	11.3	7.5	9.1	5.6	8.6	5.1	5.3
PUFA (%)		3.4	1.5	2.5	1.9	1.3	1.6	1.3	1.5	1.0	1.3	0.8	0.7
n-6 (%)		2.2	0.8	1.6	1.2	0.7	1.1	1.0	1.1	0.7	0.6	0.3	0.3
n-3 (%)		1.2	0.7	0.9	0.7	0.5	0.5	0.4	0.4	0.3	0.7	0.4	0.4
Total fatty acids (mg/100 g milk)		1434.0	409.8	1311.2	1027.6	1829.4	1626.0	3179.9	933.7	4099.1	3171.7	6473.7	9899.7

Table 3A. Continued

		Bela									
Time of Lactation (months)		**11.8**	**12.3**	**13**	**13.9**	**14.6**	**15.2**	**16.1**	**16.9**	**18.4**	**19**
Fatty acid composition (%):											
Common name:	**Abbreviation:**										
Butyric	C4:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Caproic	C6:0	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.3	0.2
Caprylic	C8:0	10.6	11.1	9.9	9.5	10.2	10.9	10.3	11.1	11.3	10.0
Capric	C10:0	57.1	59.0	61.2	61.0	61.7	62.8	62.7	63.3	61.7	62.2
Hendecanoic	C11:0	1.5	1.1	1.5	1.5	1.6	1.4	2.5	2.2	2.8	1.1
Lauric	C12:0	16.8	16.7	18.1	17.3	17.2	17.2	18.3	17.6	16.8	18.5
Tridecoic	C13:0	0.1	0.0	0.1	0.1	0.1	0.1	0.2	0.1	0.2	0.0
Myristic	C14:0	1.2	1.1	1.2	1.1	1.1	1.0	1.1	1.0	1.0	1.2
Myristoleic	C14:1c9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pentadecylic	C15:0	0.1	1.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Pentadecenoic	C15:1c10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Palmitic	C16:0	3.2	2.4	2.4	2.8	2.5	1.8	1.7	1.5	2.0	2.0
Palmitoleic	C16:1c9	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1
Margaric	C17:0	1.4	1.0	0.4	0.8	0.5	0.2	0.3	0.4	0.4	0.7
Heptadecenoic	C17:1c10	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1
Stearic acid	C18:0	1.1	0.7	0.6	0.7	0.5	0.4	0.3	0.3	0.4	0.4
Elaidic	C18:1t9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Oleic	C18:1c9	4.8	3.5	3.3	3.6	3.2	2.8	1.7	1.6	2.2	2.5
Vaccenic	C18:1c7	0.4	0.3	0.2	0.2	0.2	0.3	0.1	0.0	0.1	0.2
Linolelaidic	C18:2t9,12 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Linoleic	C18:2c9,12 (n-6)	0.6	0.9	0.3	0.4	0.4	0.3	0.3	0.4	0.4	0.4
Arachidic	C20:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
γ-Linolenic	C18:3c6,9,12 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Eicosenoic	C20:1c11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
α-Linolenic	C18:3c9,12,15 (n-3)	0.4	0.3	0.3	0.3	0.3	0.2	0.1	0.1	0.1	0.2
Conjugated linoleic acid (CLA)	C18:2c9t11 (n-6) (CLA)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Heneicosanoic	C21:0	0.4	0.3	0.0	0.2	0.1	0.0	0.0	0.0	0.0	0.2
Eicosadienoic	C20:2c11,14 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Behenic	C22:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Eicosatrienoic	C20:3c8,11,14 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Erucic	C22:1c13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Eicosatrienoic	C20:3c11,14,17 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Arachidonic	C20:4c5,8,11,14 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tricosanoic	C23:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Docosadienoic	C22:2c13,16 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Eicosopentaenoic	C20:5c5,8,11,14,17 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lignoceric	C24:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nervonic	C24:1c15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Docosapentaenoic	C22:5c7,10,13,16,19 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Docosahexanoic	C22:6c4,7,10,13,16,19 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fatty acid ratios:											
SFA (%)		93.6	94.7	95.6	95.2	95.7	96.3	97.7	97.9	96.9	96.6
MUFA (%)		5.4	4.1	3.7	4.0	3.6	3.3	1.9	1.6	2.5	2.8
PUFA (%)		1.0	1.2	0.6	0.8	0.7	0.4	0.4	0.5	0.6	0.6
n-6 (%)		0.6	0.9	0.3	0.4	0.4	0.3	0.3	0.4	0.4	0.4
n-3 (%)		0.4	0.3	0.3	0.3	0.3	0.2	0.1	0.1	0.1	0.2
Total fatty acids (mg/100 g milk)		1739.7	2397.5	9124.3	2519.9	5044.2	14500.7	9932.0	8150.6	7478.6	6330.2

Table 3B. Milk fatty acid composition of elephant Mussina and Shan at mid- and late-lactation

		Mussina				Shan	
Time of lactation (Months)		**11**	**12**	**13.5**	**14**	**21**	**22.5**
Fatty acid composition (%):							
Common name:	**Abbreviation:**						
Butyric	C4:0	0.0	0.0	0.0	0.0	0.0	0.0
Caproic	C6:0	0.0	0.0	0.0	0.1	0.0	0.0
Caprylic	C8:0	6.3	6.8	7.2	7.8	8.4	7.7
Capric	C10:0	50.5	51.1	57.4	51.5	65.6	64.1
Hendecanoic	C11:0	1.3	0.8	0.8	0.7	1.2	0.9
Lauric	C12:0	18.0	17.6	19.9	15.8	18.8	19.6
Tridecoic	C13:0	0.1	0.0	0.0	0.0	0.1	0.0
Myristic	C14:0	1.5	1.5	1.3	1.3	1.0	1.0
Myristoleic	C14:1c9	0.0	0.0	0.0	0.0	0.0	0.0
Pentadecylic	C15:0	0.1	0.1	0.4	0.1	0.4	0.4
Pentadecenoic	C15:1c10	0.0	0.0	0.0	0.0	0.0	0.0
Palmitic	C16:0	6.7	6.1	3.9	7.2	1.4	2.0
Palmitoleic	C16:1c9	0.4	0.4	0.2	0.7	0.1	0.1
Margaric	C17:0	0.6	0.5	0.4	0.7	0.3	0.4
Heptadecenoic	C17:1c10	0.1	0.1	0.0	0.2	0.0	0.0
Stearic acid	C18:0	1.1	1.0	0.6	1.0	0.4	0.4
Elaidic	C18:1t9	0.0	0.0	0.0	0.0	0.0	0.0
Oleic	C18:1c9	10.6	11.2	6.6	10.4	2.0	2.9
Vaccenic	C18:1c7	0.8	0.9	0.4	1.0	0.0	0.0
Linolelaidic	C18:2t9,12 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0
Linoleic	C18:2c9,12 (n-6)	1.0	0.8	0.3	0.7	0.3	0.3
Arachidic	C20:0	0.0	0.0	0.0	0.0	0.0	0.0
γ-Linolenic	C18:3c6,9,12 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0

Eicosenoic	C20:1c11	0.0	0.0	0.0	0.0	0.0	0.0
α-Linolenic	C18:3c9,12,15 (n-3)	0.6	0.7	0.4	0.6	0.2	0.2
Conjugated linoleic acid (CLA)	C18:2c9t11 (n-6) (CLA)	0.0	0.0	0.0	0.0	0.0	0.0
Heneicosanoic	C21:0	0.1	0.1	0.1	0.1	0.1	0.1
Eicosadienoic	C20:2c11,14 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0
Behenic	C22:0	0.0	0.0	0.0	0.0	0.0	0.0
Eicosatrienoic	C20:3c8,11,14 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0
Erucic	C22:1c13	0.1	0.1	0.0	0.0	0.0	0.0
Eicosatrienoic	C20:3c11,14,17 (n-3)	0.0	0.1	0.0	0.1	0.0	0.0
Arachidonic	C20:4c5,8,11,14 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0
Tricosanoic	C23:0	0.0	0.0	0.0	0.0	0.0	0.0
Docosadienoic	C22:2c13,16 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0
Eicosopentaenoic	C20:5c5,8,11,14,17 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0
Lignoceric	C24:0	0.0	0.0	0.0	0.0	0.0	0.0
Nervonic	C24:1c15	0.0	0.0	0.0	0.0	0.0	0.0
Docosapentaenoic	C22:5c7,10,13,16,19 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0
Docosahexanoic	C22:6c4,7,10,13,16,19 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0
Fatty acid ratios:							
SFA (%)		86.4	85.8	92.2	86.3	97.5	96.5
MUFA (%)		11.9	12.6	7.2	12.3	2.0	3.0
PUFA (%)		1.7	1.6	0.7	1.4	0.4	0.5
n-6 (%)		1.0	0.9	0.3	0.8	0.3	0.3
n-3 (%)		0.7	0.7	0.4	0.6	0.2	0.2
Total fatty acids (mg/100 g milk)		4099.8	5795.2	5399.2	4609.7	5865.3	4962.0

Table 4A. Milk total phospholipid fatty acid content of African elephant (Bela) during lactation.

								Bela	sn= 16									
Time of lactation (months)		**0.03**	**1.4**	**2**	**3.8**	**4.7**	**8**	**11.8**	**12.3**	**13.0**	**13.5**	**14.6**	**15.2**	**16.1**	**17.0**	**18.4**	**19.0**	
Fatty acid composition (%):																		
Common name:	**Abbreviation:**																	
Butyric	C4:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00 ± 0.00
Caproic	C6:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00 ± 0.00
Caprylic	C8:0	0.0	0.0	0.4	0.2	0.9	0.0	13.4	13.3	0.0	2.7	0.0	0.0	0.0	0.0	0.4	0.0	1.96 ± 4.49
Capric	C10:0	17.4	12.1	33.3	34.9	29.0	21.2	34.7	54.7	6.9	22.7	13.1	9.9	8.2	23.1	16.8	2.0	20.17 ± 14.55
Hendecanoic	C11:0	0.7	0.1	1.7	1.8	1.5	0.3	0.7	1.0	4.4	0.0	0.6	1.7	1.8	0.5	0.0	0.0	1.05 ± 1.13
Lauric	C12:0	8.8	8.2	19.3	15.0	10.1	6.1	4.9	7.4	1.5	3.9	8.1	9.9	2.1	5.6	3.6	8.6	7.68 ± 4.60
Tridecoic	C13:0	0.0	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.03 ± 0.07
Myristic	C14:0	1.6	2.9	2.5	1.9	1.7	1.2	0.9	0.5	1.2	1.1	1.6	3.8	1.4	1.8	1.7	4.0	1.85 ± 0.98
Myristoleic	C14:1c9	0.3	0.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.04 ± 0.08
Pentadecylic	C15:0	0.0	0.2	0.2	0.0	0.3	0.0	0.4	0.1	1.7	0.0	0.2	0.3	0.0	0.0	0.2	0.3	0.24 ± 0.42
Pentadecenoic	C15:1c10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00 ± 0.00
Palmitic	C16:0	15.0	17.5	8.5	9.7	10.8	14.8	7.7	3.9	20.2	13.2	16.5	22.4	25.2	19.4	21.7	27.9	15.91 ± 6.74
Palmitoleic	C16:1c9	2.2	1.6	1.4	0.6	0.8	0.4	0.5	0.3	1.8	0.3	0.9	1.6	0.0	0.0	0.0	0.5	0.81 ± 0.70
Margaric	C17:0	0.3	0.6	0.3	0.1	0.7	0.8	0.6	0.3	0.5	1.0	0.5	0.2	0.0	0.8	0.7	1.4	0.55 ± 0.35
Heptadecenoic	C17:1c10	0.3	0.4	0.0	0.4	0.6	0.0	1.3	0.4	0.1	0.5	0.2	0.1	0.8	0.6	0.9	0.0	0.41 ± 0.36
Stearic acid	C18:0	12.0	13.2	3.9	8.0	9.4	15.9	8.6	3.6	10.9	15.4	8.9	10.8	32.4	20.2	21.5	15.6	13.13 ± 7.20
Elaidic	C18:1t9	0.0	0.0	0.0	0.1	0.1	0.0	0.3	0.1	0.5	0.1	0.8	0.2	0.0	0.0	0.0	0.6	0.18 ± 0.24
Oleic	C18:1c9	31.8	32.4	23.1	21.1	26.2	24.0	19.7	11.1	30.0	25.4	37.2	26.7	14.0	20.8	22.4	19.9	24.12 ± 6.73
Vaccenic	C18:1c7	1.4	1.6	0.0	0.0	1.4	0.3	0.1	0.1	2.7	0.5	2.0	2.0	0.2	0.0	0.3	0.0	0.80 ± 0.91
Nonoadecanoic	C19:0	0.2	0.0	0.0	0.0	0.1	0.0	0.4	0.0	0.0	0.2	2.1	2.4	0.0	0.1	0.0	2.3	0.50 ± 0.89
Linolelaidic	C18:2t9,12 (n-6)	0.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.6	1.1	0.1	0.0	1.2	0.5	0.2	0.0	0.29 ± 0.41
Linoleic	C18:2c9,12 (n-6)	2.9	1.9	1.8	2.1	2.5	1.7	2.4	1.0	9.0	2.0	5.4	6.4	0.6	2.0	2.0	2.5	2.89 ± 2.19
Arachidic	C20:0	0.1	0.5	0.3	0.4	0.4	0.1	0.3	0.1	0.0	0.3	0.1	0.0	0.5	0.4	0.3	0.2	0.26 ± 0.17
g-Linolenic	C18:3c6,9,12 (n-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.1	0.4	0.7	1.1	0.0	0.18 ± 0.32

Eicosenoic	C20:1c11	0.7	0.1	0.1	0.1	0.1	0.9	0.0	0.0	0.2	1.1	0.0	0.0	0.5	0.0	0.0	0.0	0.23 ± 0.35
α-Linolenic	C18:3c9,12,15 (n-3)	2.1	0.2	1.4	1.5	0.9	0.4	1.2	0.8	0.1	0.7	0.2	0.4	0.8	0.2	0.5	0.0	0.73 ± 0.58
Conjugated linoleic acid	C18:2*c*9,*t*11(*n*-6)(CLA)	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.03 ± 0.05
Conjugated linoleic acid	C18:2*t*10,*c*12(*n*-6)(CLA)	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.04 ± 0.08
Heneicosanoic	C21:0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.04 ± 0.04
Eicosadienoic	C20:2c11,14 (n-6)	0.0	0.0	0.0	0.1	0.2	0.5	0.4	0.0	0.4	0.1	0.0	0.1	0.9	0.4	0.5	13.0	1.03 ± 3.20
Behenic	C22:0	0.1	0.1	0.0	0.1	0.0	0.2	0.0	0.0	0.0	0.2	0.1	0.0	1.0	0.3	0.3	0.0	0.14 ± 0.24
Eicosatrienoic	C20:3c8,11,14 (n-6)	0.3	0.2	0.3	0.1	0.2	0.9	0.1	0.1	0.1	0.4	0.2	0.1	0.7	0.4	0.6	0.1	0.30 ± 0.25
Erucic	C22:1c13	0.0	0.1	0.4	0.2	0.5	3.3	0.3	0.1	0.1	0.2	0.0	0.1	0.8	0.1	0.6	0.1	0.43 ± 0.81
Eicosatrienoic	C20:3c11,14,17 (n-3)	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.2	0.0	0.0	0.0	0.05 ± 0.05
Arachidonic	C20:4c5,8,11,14 (n-6)	0.2	0.2	0.1	0.1	0.5	0.4	0.6	0.3	2.8	0.3	0.6	0.1	0.2	0.5	0.2	0.1	0.45 ± 0.64
Tricosanoic	C23:0	0.3	4.6	0.1	0.8	0.5	5.4	0.0	0.5	1.4	4.5	0.2	0.1	0.2	1.0	1.2	0.1	1.30 ± 1.80
Docosadienoic	C22:2c13,16 (n-6)	0.0	0.4	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.06 ± 0.09
Eicosopentaenoic	C20:5c5,8,11,14,17 (n-3)	0.1	0.1	0.1	0.3	0.0	0.1	0.0	0.0	0.6	0.1	0.0	0.0	0.7	0.0	0.1	0.0	0.15 ± 0.20
Lignoceric	C24:0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.2	0.1	0.0	0.1	0.2	0.3	0.0	0.07 ± 0.08
Nervonic	C24:1c15	0.1	0.0	0.1	0.0	0.3	0.0	0.2	0.1	0.2	0.6	0.0	0.1	4.2	0.1	1.1	0.0	0.44 ±1.05
Docosapentaenoic	C22:5c7,10,13,16,19 (n-3)	0.2	0.1	0.0	0.1	0.0	0.8	0.4	0.0	0.9	1.0	0.1	0.1	0.5	0.2	0.8	0.0	0.33 ± 0.35
Docosahexanoic	C22:6c4,7,10,13,16,19 (n-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.3	0.0	0.1	0.2	0.05 ± 0.07
Fatty acid ratios:																		
SFA (%)		56.5	60.1	70.8	72.9	65.7	66.1	72.6	85.4	49.0	65.2	52.0	61.5	72.9	73.4	68.7	62.6	65.97 ± 9.10
MUFA (%)		36.8	36.4	25.2	22.5	30.0	29.0	22.3	12.3	35.7	28.7	41.1	30.8	20.5	21.6	25.3	21.1	27.45 ± 7.56
PUFA (%)		6.7	3.5	4.1	4.6	4.3	5.0	5.1	2.3	15.3	6.0	6.9	7.6	6.6	4.9	6.1	16.3	6.58 ± 3.85
n-6 (%)		4.4	2.7	2.5	2.8	3.4	3.7	3.5	1.4	14.2	4.1	6.5	6.9	4.7	4.4	4.6	16.0	5.36 ± 4.05
n-3 (%)		2.4	0.4	1.7	2.0	1.0	1.4	1.6	0.9	1.6	2.0	0.4	0.8	2.4	0.5	1.5	0.3	1.30 ± 0.72

Table 4B. Milk total phospholipid fatty acid composition of elephant Mussina and Shan at mid- and late-lactation.

				Mussina	sn= 4		Shan	sn= 2	
Time of lactation (months)		**11.0**	**12.0**	**13.5**	**14.0**		**21**	**22.5**	
Fatty acid composition (%):									
Common name:	**Abbreviation:**								
Butyric	C4:0	0.0	0.0	0.0	0.0	0.00 ± 0.00	0.0	0.0	0.00 ± 0.00
Caproic	C6:0	0.0	0.0	0.0	0.0	0.00 ± 0.00	0.0	0.0	0.00 0.00
Caprylic	C8:0	0.0	0.0	0.0	0.0	0.00 ± 3.93	0.0	0.0	0.00 ± 3.67
Capric	C10:0	9.7	0.6	11.8	3.1	6.30 ± 13.61	6.1	6.6	6.34 ± 12.99
Hendecanoic	C11:0	2.2	0.8	1.4	1.9	1.56 ± 1.00	2.6	0.2	1.43 ± 0.99
Lauric	C12:0	3.0	0.6	3.4	0.7	1.93 ± 4.55	2.4	8.3	5.36 ± 4.4
Tridecoic	C13:0	0.0	0.0	0.0	0.0	0.00 ± 0.06	0.0	0.0	0.00 ± 0.06
Myristic	C14:0	1.3	1.5	1.1	1.1	1.24 ± 0.88	1.6	4.1	2.85 ± 0.95
Myristoleic	C14:1c9	0.7	0.0	0.8	0.5	0.51 ± 0.24	0.0	0.2	0.08 ± 0.24
Pentadecylic	C15:0	0.0	0.0	0.0	0.0	0.00 ± 0.37	0.0	0.3	0.16 ± 0.35
Pentadecenoic	C15:1c10	0.0	0.0	0.0	0.0	0.00 ± 0.00	0.0	0.0	0.00 ± 0.00
Palmitic	C16:0	22.0	24.7	18.0	21.2	21.46 ± 6.56	21.3	25.5	23.42 ± 6.72
Palmitoleic	C16:1c9	0.5	2.2	0.3	0.6	0.90 ± 0.68	0.7	1.7	1.24 ± 0.65
Margaric	C17:0	0.5	0.3	0.6	0.6	0.50 ± 0.31	0.7	0.6	0.63 ± 0.29
Heptadecenoic	C17:1c10	1.3	0.3	1.0	1.3	0.98 ± 0.42	0.5	0.0	0.24 ± 0.41
Stearic acid	C18:0	22.5	12.2	15.1	24.7	18.64 ± 7.00	15.0	10.5	12.74 ± 6.67
Elaidic	C18:1t9	0.4	0.0	0.0	0.1	0.13 ± 0.22	0.1	0.0	0.08 ± 0.20
Oleic	C18:1c9	24.3	39.1	26.4	32.2	30.50 ± 7.99	28.2	32.0	30.12 ±8.15
Vaccenic	C18:1c7	0.4	3.0	0.8	0.6	1.19 ± 0.91	2.1	0.0	1.06 ± 0.89
Nonoadecanoic	C19:0	0.3	0.0	0.0	0.4	0.17 ± 0.78	0.2	3.3	1.71 ± 0.90
Linolelaidic	C18:2t9,12 (n-6)	1.3	0.0	1.9	2.0	1.30 ± 0.63	0.0	0.0	0.00 ± 0.62
Linoleic	C18:2c9,12 (n-6)	0.1	10.9	1.0	0.8	3.19 ± 2.69	7.4	5.3	6.37 ± 2.66
Arachidic	C20:0	0.1	0.2	0.2	0.2	0.19 ± 0.91	0.3	0.1	0.19 ± 0.14

g-Linolenic	C18:3c6,9,12 (n-6)	2.2	0.1	1.7	1.3	1.35 ± 0.63	0.6	0.0	0.32 ± 0.61
Eicosenoic	C20:1c11	0.0	0.0	0.0	0.0	0.00 ± 0.31	0.0	0.1	0.06 ± 0.29
α-Linolenic	C18:3c9,12,15 (n-3)	0.9	0.6	1.5	0.1	0.76 ± 0.54	1.2	0.1	0.69 ± 0.52
Conjugated linoleic acid	C18:2*c*9,*t*11(*n*-6)(CLA)	0.2	0.1	0.0	0.3	0.14 ± 0.07	0.0	0.0	0.00 ± 0.07
Conjugated linoleic acid	C18:2*t*10,*c*12(*n*-6)(CLA)	0.5	0.0	2.9	0.2	0.91 ± 0.62	0.0	0.0	0.02 ± 0.59
Heneicosanoic	C21:0	0.2	0.1	0.9	0.0	0.32 ± 0.20	0.0	0.0	0.00 ± 0.19
Eicosadienoic	C20:2c11,14 (n-6)	0.3	0.3	0.9	0.6	0.50 ± 2.76	0.9	0.1	0.47 ± 2.56
Behenic	C22:0	0.1	0.1	0.5	0.0	0.16 ± 0.22	0.3	0.0	0.15 ± 0.21
Eicosatrienoic	C20:3c8,11,14 (n-6)	1.9	0.2	0.3	0.4	0.70 ± 0.41	0.0	0.1	0.03 ± 0.39
Erucic	C22:1c13	0.1	0.1	0.8	0.6	0.38 ± 0.70	0.5	0.1	0.27 ± 0.65
Eicosatrienoic	C20:3c11,14,17 (n-3)	0.1	0.1	0.1	0.9	0.31 ± 0.19	0.2	0.0	0.09 ± 0.18
Arachidonic	C20:4c5,8,11,14 (n-6)	0.1	1.0	0.2	0.6	0.48 ± 0.57	3.0	0.6	1.84 ± 0.73
Tricosanoic	C23:0	0.1	0.1	2.7	0.1	0.77 ± 1.61	0.8	0.0	0.42 ± 1.50
Docosadienoic	C22:2c13,16 (n-6)	0.1	0.0	0.0	0.7	0.20 ± 0.16	0.0	0.0	0.01 ± 0.15
Eicosopentaenoic	C20:5c5,8,11,14,17 (n-3)	0.1	0.0	0.1	0.2	0.09 ± 0.17	0.0	0.0	0.03 ± 0.16
Lignoceric	C24:0	0.3	0.1	0.3	0.2	0.22 ± 0.09	0.2	0.0	0.09 ± 0.09
Nervonic	C24:1c15	0.9	0.6	0.6	0.2	0.58 ± 0.91	2.1	0.0	1.05 ± 0.90
Docosapentaenoic	C22:5c7,10,13,16,19 (n-3)	1.5	0.0	2.5	1.5	1.39 ± 0.64	0.8	0.0	0.41 ± 0.62
Docosahexanoic	C22:6c4,7,10,13,16,19 (n-3)	0.0	0.1	0.0	0.1	0.05 ± 0.06	0.1	0.0	0.06 ± 0.06
Fatty acid ratios:									
SFA (%)		62.1	41.3	56.1	54.2	53.45 ± 15.14	51.4	59.5	55.47 ± 16.63
MUFA (%)		28.7	45.2	30.8	36.1	35.18 ± 8.86	34.3	34.1	34.20 ± 9.26
PUFA (%)		9.2	13.5	13.1	9.7	11.37 ± 3.93	14.3	6.4	10.33 ± 4.00
n-6 (%)		6.6	12.6	9.0	6.5	8.67 ± 3.84	12.0	6.2	9.08 ± 3.78
n-3 (%)		2.6	0.8	4.2	2.7	2.60 ± 0.97	2.3	0.2	1.28 ± 0.97

Table 5A. Milk sterol content of African elephant (Bela) during lactation

Months	Campesterol (µM)	Cholesterol (mM)	Lathosterol (µM)	Sitosterol (µM)
9.2	2.7	2.1	4.8	
10.2	2.8	2.2	4.7	
11.8	6.1	3.8	5.5	7.1
12.3	17.4	13.3	7.2	9.6
13	7.2	6.7	5.6	6.5
13.9	11.4	5.3	5.4	7.3
15.2	9.4	7.4	6.4	9.2
15.8	3.7	0.9	4.1	5.3
16.9	10.6	8.9	5.7	6.9
18.4	19.6	11.9	5.9	6.9
19	2.7	0.3	4.0	5.4
sn= 11	8.5 ± 5.9	5.9 ± 5.1	5.4 ± 1.0	7.1 ± 1.5

Table 5B. Milk sterol content of African elephant (Mussina) at mid-lactation lactation

Months	Campesterol (µM)	Cholesterol (mM)	Lathosterol (µM)	Sitosterol (µM)
11	2.9	2.2	4.2	5.5
12	12.9	1.9	4.2	8.1
13.5	2.5	0.5	3.8	5.5
14	3.4	2.2	3.8	4.5
sn= 4	5.4 ± 5.0	1.7 ± 0.8	4.0 ± 0.2	5.9 ± 1.5

Table 6A. Changes in calculated and measured gross energy of African elephant milk (Bela) during lactation

Months	Energy KJ	Calories KCal	GE (general Energy)	Gross Energy
0.03	124.7	29.8	67.6	47.6
2	125.1	29.9	79	34
2.8	152.4	36.4	82.8	44.7
3.8	117.9	28.2	58.3	45.3
4.7	145.5	34.7	86.8	50.7
6	125.7	30	77.4	35.7
8	147.4	35.2	102.1	65.9
9.2	288.8	69	124.9	90.3

10.2	255.1	60.9	156	123.1
12	117.9	28.2	58.3	45.3
13	275.6	65.8	174.7	143.3
13.9	241.2	57.6	154.2	124.5
14.6	361.7	86.4	132.4	98.8
16.1	242.2	57.8	199.9	169.3
16.9	362	86.5	174.3	145
22.5	299	71.6	171.8	145.7

Table 6B. Changes in calculated and measured gross energy of elephant milk (Mussina and Shan) at mid- and late-lactation

Months	**Energy KJ**	**Calories KCal**	**GE (general Energy)**	**Gross Energy**
11	240.4	57.4	119.8	87.1
12	191.1	45.6	151.5	118.7
13.5	171.2	40.9	137.5	108.7
14	212.6	50.8	125.5	97.4
22.5	299	71.6	171.8	145.7