The milk and serum NMR-based metabolic profiles of the South African giraffe (*Giraffa camelopardalis giraffa*) and their relation to other milk nutrients

Lauren Lorraine Schmidt

Thesis submitted for the degree: **Master of Science in Food Science**

Thesis submitted in fulfilment of the requirements in respect of the Master's degree Master of Science in Food Science in the Department of Microbial, Biochemical and Food Biotechnology, Faculty of Natural and Agricultural Sciences, University of the Free State.

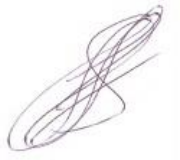
Supervisor: **Prof. Gernot Osthoff**

Co-supervisor: **Prof. Adrian S.W. Tordiffe**

29 January 2020

Declaration

I, Lauren Lorraine Schmidt, declare that the Master's Degree research dissertation that I herewith submit for the Master of Science in Food Science degree at the University of the Free State is my own independent work and that I have not previously submitted it for a qualification at another institution of higher education.



Lauren Schmidt

Acknowledgements

This research was supported by the National Research Foundation and University of the Free State. I would like to thank Professor Osthoff for his patience and time from the beginning of my thesis up until its completion. I would also like to thank Professor Tordiffe for sharing his knowledge on metabolites.

Summary

There is little information available on the milk nutrients of wild mammals and none on their milk and serum metabolomes. The giraffe is the largest ruminant and this study compares the information obtained from giraffe with the most common domesticated ruminant, the cow. This is because a lot of information on the cow is available in literature. What makes this study interesting is that the pregnancy status of the female giraffes could be investigated as a variable. The giraffe is one of very few species that can fall pregnant while simultaneously caring for a calf.

The current study is part of a larger project regarding behaviour, ecology and biology of giraffes in the Rooipoort Nature Reserve near Kimberley in the Northern Cape. Giraffes were sedated to fit radio transmitters in 2017, and again in 2018 for the removal. Simultaneously, biological samples were collected, inter alia blood and milk. Blood and milk was collected from eleven female giraffes and blood from nine male giraffes. Blood and milk was also obtained from two females in the Sandveld Nature Reserve, Hoopstad district, Free State Province. The metabolites in serum and milk were analysed by Nuclear Magnetic Resonance (NMR) spectroscopy and the milk nutrients (proteins, carbohydrates, fats and fatty acids, and minerals) were analysed according to standard procedures.

This study presents a baseline metabolome of serum and milk for giraffes. The results showed differences in serum metabolite concentrations between the 2017 and 2018 samples which could be due to a variation in rainfall and the related availability of browse at the time of collection. The 2018 giraffe group milk metabolite concentrations, particularly lactose, suggest that they were on a lower plain of nutrition when compared to the 2017 giraffe group. The results showed a variation in milk nutrient concentrations between giraffes of different locations and this may be due to different browse being available at these locations because the nutrient content of the browse influences the milk nutrient concentrations.

Statistical analysis of milk and serum metabolites showed the greatest differences were between the years of sampling rather than pregnancy status. Pregnancy status was not a significant contribution to the differences in milk nutrients and metabolites.

A correlation analysis was used to evaluate the relationship between serum and milk metabolites of female giraffes and their milk nutrients. Milk metabolites were more strongly correlated with milk nutrients than serum metabolites. The serum amino acids isoleucine and valine were positively correlated with milk total protein and casein protein. The milk

metabolites alanine, isoleucine, leucine, phenylalanine, and valine were positively correlated with milk whey protein.

The serum amino acids isoleucine, leucine, phenylalanine, tyrosine, and valine are all negatively correlated with milk lactose. The concentrations of these amino acids are higher for the 2018 group and the milk lactose concentration is lower for the 2018 group. The lower energy intake for the 2018 group is responsible for the lower lactose concentration and this is correlated with the higher concentrations of serum amino acids because serum amino acids, specifically branched chain amino acids increase in concentrations during starvation.

Diet is likely responsible for the positive correlations of milk creatine and creatinine with calcium, iron, lactose, butyric acid, caproic acid, and linoleic acid. Diet affects the concentrations of metabolites and nutrients in milk.

Milk citric acid cycle metabolites are positively correlated with lactose. A more energy dense diet, as proposed for the 2017 giraffe group, would lead to an increase in glucose production making more glucose available for lactose production as well as more glucose available to enter the citric acid cycle.

KEY WORDS

Giraffe, *Giraffa camelopardalis*, milk nutrients, milk metabolites, serum metabolites, NMR metabolomics

Table of Contents

Chapter 1 Literature Review 12

1.1 Introduction 12

1.2. The giraffe 13

1.3. Metabolism 13

1.3.1. Metabolites 13

1.3.2. Metabolic fuel 13

1.3.3. Metabolite production 14

1.3.4. Carbohydrate metabolism and metabolites 14

1.3.5. Lipid metabolism and metabolites 15

1.3.6. Protein metabolism and metabolites 16

1.3.7. Metabolism during the transition period in the cow 17

1.3.8. Foetal-Neonatal nutrition 19

1.3.9. Nutrimetabolomics 20

1.4. Serum metabolic studies on cows 20

1.4.1. Cow 21

1.4.2. Pregnant dairy cow 22

1.5. Interspecies comparison of milk metabolites 22

1.5.1. Metabolome of cow's milk 23

1.5.2. Metabolome of goat's milk 24

1.5.3. Metabolome of giant panda milk 24

1.5.4. Metabolome of donkey milk 25

1.5.5. Metabolome of human milk 25

1.6. Nutrient composition of milk 25

1.6.1. Carbohydrates 25

1.6.2. Fat and fatty acids 26

1.6.3. Proteins 27

1.6.4. Interspecies comparison of milk nutrients 28

1.7. Research of milk of wild mammals 30

1.8. Discussion 30

1.9. Aims 31

Chapter 2 - Metabolites in giraffe serum 32

2.1. Introduction 32

2.2. Materials and methods 33

2.2.1. Study site and sample collection 33

2.2.2. Sample preparation for NMR analysis 33

2.2.3. ^{1}H-NMR analysis 34

2.2.4. Statistical analysis 34

2.2.5. MetaboAnalyst pathway analysis 34

2.3. Results 35

2.4. Discussion 43

2.4.1. Serum metabolome differences based on sex. 43

2.4.2. Serum metabolome differences based on year - nutrition 44

2.4.3. Serum metabolome differences based on year – temperature 45

2.4.4. Serum metabolome differences based on year – energy deficiency 46

2.4.5. Serum metabolome comparison between species 47

2.5. Conclusion 48

Chapter 3 - Metabolites in giraffe milk 49

3.1. Introduction 49

3.2. Materials and methods 49

3.2.1. Study site and sample collection 49

3.2.2. Sample preparation for NMR analysis 50

3.2.3. ^{1}H-NMR analysis 50

3.2.4. Statistical analysis 50

3.2.5. MetaboAnalyst pathway analysis 50

3.3. Results 50

3.4. Discussion 55

3.4.1. Milk metabolome differences based on year 55

3.4.2. Individual variation 57

3.4.3. Inter-species comparison of milk metabolites 57

3.4.4. Mammary gland metabolism 58

3.5. Conclusion 59

Chapter 4 - Nutrient composition of giraffe milk and its relationship to serum and milk metabolomes 61

4.1. Introduction 61

4.2. Materials and methods 61

4.2.1. Study site and sample collection 61

4.2.2. Protein fractionation 61

4.2.2.1. TN (Total Nitrogen) Determination 62

4.2.2.2. NPN (Non-protein Nitrogen) Determination 62

4.2.2.3. Whey Protein Determination 62

4.2.3. Lipid extraction and fatty acid analysis 62

4.2.4. Carbohydrate analysis 63

4.2.5. Analysis of moisture, ash and minerals 64

4.2.6. Statistical analysis 64

4.3. Results 64

4.4. Discussion 72

4.4.1. Variation of nutrient concentrations 72

4.4.3. Milk nutrients of giraffes in different locations 72

4.4.4. Factors affecting the nutrient content of giraffe milk..... 73

4.4.5. Correlation of serum and milk metabolites with milk nutrients..... 76

4.5. Conclusion 80

Chapter 5 – Concluding discussion 81

Chapter 6 - References..... 85

Chapter 7 - Appendix A..... 99

Chapter 8 - Appendix B..... 106

Chapter 9 – Appendix C..... 126

List of tables

Table 1.1: Content of macronutrients and selected fatty acids in the milk of mammals representative of different taxonomic order……29

Table 2.1. Averages of the metabolites (μM) in the serum of giraffes……37

Table 3.1: Metabolites (μM) in the milk of giraffes……52

Table 4.1: Major nutrients (g/100g) in giraffe milk according to pregnancy status, origin and year of collection……64

Table 4.2: Fatty acid content (% of total) of giraffe milk according to pregnancy status, origin and year of collection……64

Table 4.3: Minerals (mg/100g) in giraffe milk according to pregnancy status, origin and year of collection……67

Table 4.4: Microsoft Excel correlation of giraffe milk and serum metabolites with giraffe milk nutrients……68

Table A 1: Detected metabolites associated with glycolysis, the krebs cycle, gluconeogenesis, pentose phosphate pathway, and energy metabolism in the milk of mammals representative from different taxonomic orders……98

Table A 2: Metabolites relevant to amino acid metabolism, nucleic acid related metabolism, and nitrogen balance in the milk of mammals representative from different taxonomic orders……99

Table A 3: Metabolites relevant to saccharides, sugar alcohols, and related molecules in the milk of mammals representative from different taxonomic orders……101

Table A 4: Metabolites associated with organic acids and related molecules in the milk of mammals representative from different taxonomic orders……102

Table A 5: Metabolites associated with fatty acids and fatty acid derivatives in the milk of mammals representative from different taxonomic orders……103

Table A 6: Metabolites associated with vitamins in the milk of mammals representative from different taxonomic orders……104

Table B 1: Giraffe sample information……105

Table B 2: p-values based on sex and year of serum metabolites in male and female giraffes. p values based on year include only the giraffes located in Rooiport game reserve……106

Table B 3: p-values based on pregnancy status and year of serum metabolites in female giraffes. p values based on year include only the giraffes located in Rooiport game reserve……107

Table B 4: p-values based on pregnancy status and year for giraffe milk metabolites. p values based on year include only the giraffes located in Rooiport game reserve……109

Table B 5: p-values for giraffe milk nutrients. p values based on year include only the giraffes located in Rooiport game reserve……111

Table B 6: Comparison of metabolites (µM) in the serum of giraffes and humans……113

Table B 7: Metabolites (µM) in the milk of giraffes compared to Brown Swiss cows and Simmental cows……115

Table B 8: Comparison of nutrients between the giraffe averages obtained in this study with that of ruminants from literature……119

Table C 1: Rainfall (mm) at weather stations surrounding Rooiport reserve in 2017……125

Table C 2: Rainfall (mm) at weather stations surrounding Rooiport reserve in 2018……125

Table C 3: Average temperatures (°C) at weather stations surrounding Rooiport reserve in 2017…126

Table C 4: Average temperatures (°C) at weather stations surrounding Rooiport reserve in 2018…126

List of figures

Figure 2.1 ^{1}H-NMR spectra of giraffe serum……36

Figure 2.2 MetaboAnalyst pathway analysis plot based on the significant metabolites for sex showing the most preferable pathway……40

Figure 2.3 MetaboAnalyst pathway analysis plot based on the significant metabolites for sex showing the second most preferable pathway……40

Figure 2.4: MetaboAnalyst pathway analysis plot based on the significant metabolites for year (Rooiport male and females) showing the most preferable pathway……41

Figure 2.5: MetaboAnalyst pathway analysis plot based on the significant metabolites for year (Rooiport male and females) showing the second most preferable pathway……41

Figure 2.6: MetaboAnalyst pathway analysis plot based on the significant metabolites for year (Rooiport females) showing the most preferable pathway……42

Figure 3.1 ^{1}H NMR spectra for giraffe milk……51

Figure 3.2 MetaboAnalyst pathway analysis plot for the most significantly different milk metabolites (p=0.0528) for year of sampling……54

Figure B 1: Principal component analysis (PCA) plot for giraffe serum metabolites based on sex……..120

Figure B 2: Principal component analysis (PCA) plot for male and female Rooiport giraffe serum metabolites based on year……..120

Figure B 3: Principal component analysis (PCA) plot for Rooiport giraffe serum metabolites based on year for females……..120

Figure B 4: Principal component analysis (PCA) plot for giraffe serum metabolites based on pregnancy status……121

Figure B 5: Principal component analysis (PCA) plot for giraffe milk metabolites based on pregnancy status……122

Figure B 6: Principal component analysis (PCA) plot for Rooiport giraffe milk metabolites based on year……122

Figure B 7: Principal component analysis (PCA) plot for giraffe milk nutrients based on pregnancy status……123

Figure B 8: Principal component analysis (PCA) plot for Rooiport giraffe milk nutrients based on year……123

Chapter 1 Literature Review

1.1 Introduction

The giraffe (*Giraffa*) is the world's largest ruminant (Shorrocks, 2016). Female giraffes are capable of nurturing a young growing calf while being pregnant at the same time and this can increase the nutritional demands on the giraffe (Deacon et al., 2015). The giraffe gestation period is around 15 months (Shorrocks, 2016) and their lactation period is 10 to 16 months (Cavendish, 2010).

The major macronutrients in milk are proteins, carbohydrates, and fats (Kon & Cowie, 2016). Milk nutrients are synthesized in the mammary gland, but some nutrients such as short chain fatty acids are obtained directly from the blood (Palmquist, 2006; Frandson et al., 2009). The nutrient concentrations of milk varies between species (Markiewicz-Keszycka et al., 2013; Murgia et al., 2016; Osthoff et al., 2017).

Metabolites are biochemical molecules involved in interactions concerning energy provision, growth, and reproduction (Bagchi et al., 2015). The complete set of metabolites from a biofluid is known as a metabolome. The metabolomes bodily fluids can be influenced by factors such as status of diet and health as well as gut microbiota (Lämmerhofer & Weckwerth, 2013).

The major nutrients namely, protein, carbohydrates, and fats, are all metabolised differently and produce a variety of different metabolites. The study of metabolites is a useful tool for nutrition research and the detection of disorders and diseases (Lämmerhofer & Weckwerth 2013; Bagchi et al. 2015). The type and amount of metabolites in milk have been shown to indicate metabolic disorders (Bezerra et al., 2014) and have been reported to be correlated to certain milk traits such as protein content (Melzer et al., 2013). Blood metabolites are transferred to the mammary cells in order to be used up for milk nutrient synthesis, while some, such as citrate, free fatty acids, amino acids and monosaccharides can also be found in the milk (Frandson et al., 2009).

Certain metabolites, such as blood glucose and ketone bodies are related to the energy status of the mammal (Erfle et al., 1974) and the higher energy demand during lactation can influence the concentrations of metabolites present in biofluids (Kara et al., 2013). A different array of metabolites is present in the milk of different species (Klein et al., 2010; Melzer et al., 2013; Caboni et al., 2016). Diet (Tordiffe et al., 2016) and pregnancy (Guo & Tao, 2018) can influence the concentrations of metabolites.

1.2. The giraffe

Giraffes are the world's largest ruminants (Shorrocks, 2016) and are classified as concentrate selectors (browsers) which means they selectively browse trees and bush foliage (Valdes & Schlegel, 2012). Giraffes favour the leaves of the Acacia tree (Parker, 2005). There are nine subspecies of giraffes (Parker, 2005) and they have been listed as 'vulnerable' by the International Union for Conservation of Nature (IUCN) in the Red List of Threatened Species. The gestation period for a giraffe is around 15 months (Shorrocks, 2016) and giraffe calves suckle for 10 to 16 months (Cavendish, 2010). Giraffe calves can be hand-reared using whole cow's milk and it is recommend that the calf be weaned at 1 year of age (Casares et al., 2012). Giraffes can nurture a foetus and a young growing calf as a simultaneous reproductive strategy. Giraffes are one of the few species of larger mammals that can do this. This kind of reproductive strategy places increased nutritional demands on the giraffe and can cause a reduced nutritional status (Deacon et al., 2015). It would be interesting to find out whether the blood and milk metabolomes as well as the milk nutrients are affected by pregnancy status.

1.3. Metabolism

This section summarizes the metabolic processes of energy production and gives an overview of how major nutrients are catabolised. The effect of pregnancy and lactation on energy metabolism is also described.

1.3.1. Metabolites

Metabolism is an incredibly dynamic process and there is a rapid turnover of metabolic intermediates. Metabolites are defined as those molecules which are involved in interactions at the biochemical level, and they provide the energy and the building blocks for the synthesis of structural components of other biomolecules, which are required for growth and reproduction. The complete set of metabolites is known as the metabolome (Bagchi et al., 2015; Nielsen & Jewett, 2007; Lämmerhofer & Weckwerth, 2013). Interaction of metabolites with enzymes form a complex metabolic network known as metabolism. (Bagchi et al. 2015).

1.3.2. Metabolic fuel

All metabolic processes require metabolic energy in the form of adenosine triphosphate (ATP). The cells need to release the electrons present in food in order to yield ATP. Carbohydrates, proteins, and lipids go through metabolic processes that all lead to the production of acetyl-

CoA. Acetyl-CoA will then enter the citric acid cycle which powers the electron transport chain to produce ATP (Campbell & Farrell, 2012). During fasting, fatty acids from mobilised fat stores are utilized as fuel for beta-oxidation and ATP production. Plasma free fatty acid concentrations increase steadily by enhanced lipolysis when fasting continues. Plasma glucose is another fuel and is maintained by breakdown of liver glycogen or hepatic gluconeogenesis. Some cells, such as red blood cells, rely on glucose as a sole energy substrate. This fasting state also causes protein degradation in muscle, and amino acids are released as a third form of fuel into the plasma at varying amounts (Lämmerhofer & Weckwerth, 2013; Bagchi et al., 2015).

1.3.3. Metabolite production

Blood, milk, and urine metabolomes are integrated. This is because the metabolites present in blood can be deposited in the urine, and can also be transferred to milk. Nutrients and metabolites provided by the diet, metabolites produced endogenously in the inter-organ metabolism endogenously, as well as the metabolites produced by the microbiota in the large intestine (Lämmerhofer & Weckwerth, 2013; Bagchi et al., 2015) compose these metabolomes. Ruminants such as cows and goats have rumen bacteria and other microorganisms which also contribute to the body fluid metabolomes, specifically monosaccharides and short chain organic acids (de Almeida et al., 2018). Metabolites from the rumen can be transferred to the blood (O'Callaghan et al., 2018) and can then end up in the milk (Frandson et al., 2009).

Continuous change is a distinct feature of the metabolome from human bodily fluids (Lämmerhofer & Weckwerth, 2013; Bagchi et al., 2015). However, the blood metabolome is an exception. Metabolites such as amino acids and glucose in blood are continuously regulated due to homeostasis. Interorgan amino acid transport is an important component involved in maintaining amino acid homeostasis (Brosnan, 2003) and pancreatic regulation is responsible for glucose homeostasis (Röder et al., 2016).

1.3.4. Carbohydrate metabolism and metabolites

When carbohydrates are consumed in the form of starch, the digestive system utilises amylases to break these polysaccharides down into smaller components. Salivary alpha-amylases begin cleaving the glycosidic linkages. Further cleavage into trisaccharides and

disaccharides is completed by pancreatic alpha amylases. Maltase breaks down maltose (disaccharide) into its glucose constituents. Alpha-glucosidase cleaves maltotriose (trisaccharide) and alpha-dextrinase breaks down dextrin into the glucose constituents. Through glycolysis, glucose is converted into two pyruvate molecules in the cytoplasm of a cell. The pyruvate molecules are transported into the mitochondrial matrix of the cell where they are converted into two acetyl-CoA molecules via a decarboxylation reaction. The acetyl-CoA molecules then enter the Krebs cycle. The electrons produced from the Krebs cycle are transported via electron carrier molecules to the inner mitochondrial membrane where the electron transport chain is located and ATP molecules are produced (Campbell & Farrell 2012).

Carbohydrate metabolism is different in ruminants because they have rumen bacteria and microorganisms which ferment the plant components from their diet. Ruminants do not produce the enzyme cellulase to break down the cellulose components of a plant cell wall. Rumen bacteria and microbes produce cellulase which then digests the cellulose to produce monosaccharides and simple polysaccharides. Upon further microbe digestion, volatile fatty acids are produced. These volatile fatty acids are acetic acid, propionic acid, and buyric acid. Propionic acid is mainly used for glucose production in the liver (Frandson et al., 2009). Acetic acid can be oxidized to produce ATP and it is the major source of acetyl CoA for milk fat synthesis (Perry, 1980; J. Moran, 2005). Butyric acid is also used for milk fat synthesis (Barbosa-Cánovas et al., 2006; Christie, 2014).

1.3.5. Lipid metabolism and metabolites

Lipid digestion begins with the secretion of lingual lipase, followed by the secretion of gastric lipase. Emulsification occurs in conjuction with bile salts and peristalsis. Enzymatic digestion is completed by pancreatic enzymes, including pancreatic lipase, as well as lysophospholipase, colipase, cholesterol esterase and phospholipase A_2. Re-synthesis of triacylglycerols occurs once there is diffusion of the lipolysis products into the intestinal epithelial cells. These triacylglycerols are packed into lipid-protein complexes known as lipoproteins that transport the triacylglycerols from the intestine to the necessary adipose, muscle, and cardiac tissue. The triacylglycerols are stored as is, and when blood glucose has decreased, they can be used for energy production (Champe et al., 2005; Vance & Vance, 2008; Campbell & Farrell, 2012). Fatty acids are catabolised through fatty acid β-oxidation and protein transporters allow the fatty acids to enter the cell. Once inside the cell, they can be used for energy production.

Triacylglycerols must be mobilised from fat stores in order to be used for energy production. This process requires the hydrolytic release of glycerol and fatty acids from their triacylglycerol form by hormone-sensitive and other specific lipases. The glycerol is transported to the liver through the blood where it is then phosphorylated. Pyruvate is produced from the glycerol phosphate and can then be used in the Krebs cycle. The free fatty acids are transported as fatty acid-albumin complexes. Upon contact with the cell, the fatty acids dissociate from albumin and are taken up by the cells to be oxidised for energy production in the peroxisomes and mitochondria. Very long chain fatty acids are oxidized in the peroxisome. The shortened fatty acids can then undergo β-Oxidation which involves preliminary processes (Champe et al., 2005; Vance & Vance, 2008).

In ruminants, lipids are hydrolysed to free fatty acids in the rumen by the rumen bacteria and microbes. These free fatty acids pass out of the rumen into the small intestine. They are then converted to triglycerides and transporters deliver these triglycerides to tissues. They can be taken up by the mammary gland to produce milk fat or they can be used by other bodily tissues to produce energy (Frandson et al., 2009).

1.3.6. Protein metabolism and metabolites

Upon consumption, proteins are catabolised. Amino acids are not stored by the body. Free amino acids are taken up by the enterocytes. Peptides are hydrolysed in the enterocyte cytosol. The resulting amino acids are released into the portal vein system. The amino acids will either be metabolized by the liver or released from the portal vein into the general circulation. Transaminases are broadly distributed in human tissue. Transaminases are mostly active in the liver, kidney, skeletal muscle, and heart muscle (Bhagavan et al., 2011). The α-amino groups are removed by transamination and then oxidative deamination. The nitrogen from α-amino acids is either incorporated into other compounds or excreted. Ammonia and α-keto acids are the resulting products. Most of the ammonia produced is used to synthesise urea. The rest of the ammonia is excreted in the urine. The α-keto acids are converted into energy producing intermediates that can be metabolized into carbon dioxide and water, fatty acids, or ketone bodies. Catabolism of glucogenic amino acids produce pyruvate or oxaloacetate, the first molecule in gluconeogenesis. Leucine and lysine are ketogenic, and catabolism of these amino acids produce acetyl-CoA or acetoacetyl-CoA which lead to the production of ketone bodies. Neither of these can bring about the production of glucose. Tryptophan, isoleucine, threonine, phenylalanine, threonine, and tyrosine are categorised as glucogenic and ketogenic and catabolism of these amino acids produce both fatty acid and

glucose precursors. The reduced carbon skeleton of amino acids can be used for energy production during times of starvation (Campbell & Farrell 2012).

In ruminants, dietary protein is digested by the rumen bacteria and microorganisms. The protein is first broken down into peptides. These peptides are then broken down into invidual amino acids and eventually ammonia. The amino acids can be used to produce microbial proteins to promote microbial growth and during digestive contractions, some of these microbes enter the abomasum and the remainder of the digestive tract where they are digested similarly to the non-ruminant digestion of protein (Frandson et al., 2009).

1.3.7. Metabolism during the transition period in the cow

Giraffes can nurture a foetus and a young growing calf as a simultaneous reproductive strategy. This kind of reproductive strategy places increased nutritional demands on the giraffe and can cause a reduced nutritional status (Deacon et al., 2015), which in turn can affect their energy balance. In cows it was observed that energy imbalances can cause temporary metabolic disorders (Bezerra, et al., 2014). It is important to discuss the reasons behind the energy imbalance seen in cows because these pregnant giraffes may experience a similar imbalance.

The transition period in cattle is the period from three weeks prior to calving until three weeks postpartum. In the first week postpartum, initiation of lactation is well-indicated by the presence of the following blood parameters: non-esterified fatty acids and ketone bodies. Endocrine and metabolic alterations, lactogenesis, and foetal needs increase the mammal's energy requirements. A mammal's metabolism can compensate by making use of body reserves when there is an imbalance of energy, for a short period. This imbalance can affect the metabolite balance in both blood and milk. During a severe and persistent imbalance of energy, the mammal's body depletes its reserves and a temporary metabolic disorder will occur (Bezerra, et al., 2014). If the stress response is non-adaptive, mortality can occur (Sundrum, 2015).

At the stage of lactation, receiving of nutrients by the mammary gland is a priority. Normal dietary intake cannot provide sufficient nutrients required for lactation; thus, negative energy balance is observed (Kara et al., 2013). The rapid increase in the demand for energy exceeds the increase in food intake (Sundrum, 2015). This causes a change in blood metabolites. There is a risk of the development of ketosis in dairy cows during the transition period.

1.3.7.1. Ketosis

Ketosis is a metabolic disease characterised by an increase in concentration of ketone bodies. During the high energy demand, fatty acids are released from body fat stores and undergo esterification in the liver to form fatty acyl coenzyme A (fatty acyl CoA). Oxidation of fatty acyl CoA in the mitochondria produces ketone bodies. Ketosis occurs when the energy demands become excessive (Brown et al., 2017). Ketosis causes fat to accumulate in the liver and a fatty liver can interfere with glucose production and this can cause hypoglycaemia to ensue (Sundrum, 2015).

Metabolic stress can place the cow at risk of developing a disease such as ketosis (Kara et al., 2013). A negative energy balance in cows during the transition period can be prevented by maximizing energy intake (Randhawa et al., 2014).

1.3.7.2. Energy balance

A higher energy demand occurring during the transition period of a dairy cow can cause susceptibility to metabolic diseases such as ketosis and hepatic lipidosis. Towards the end of gestation, specifically throughout the last two to four weeks, energy requirements increase substantially during foetal development and colostrum synthesis. There is also a decrease in the consumption of dry matter. These two factors are the frequent cause of negative energy balance that develops a few weeks before parturition (Bezerra et al. 2014; Currie 1992).

Food energy deficits are compensated for by body fat mobilisation. Unfortunately, reproductive problems and disease can occur when there is an excess of fat mobilisation. For example, ketosis and Fatty Liver Syndrome can occur two to seven weeks after parturition due to excessive negative energy balance. The negative energy balance will promote adipose fat mobilisation and release of triglycerides into the blood stream (Bezerra et al. 2014; Currie 1992). Hormone sensitive lipase hydrolyses these triglycerides. Triglyceride hydrolysis produces glycerol and non-esterified fatty acids as final products that are transported by the blood to reach the liver. These non-esterified free fatty acids are taken up by hepatocytes (cells of liver's main parenchymal tissue) and esterified back to triglycerides (Campbell & Farrell 2012). Ketosis occurs as the triglycerides are transformed into ketones. Ketones are transformed into ketone bodies through ketogenesis, and the acetone and acetoacetic acid can either undergo decarboxylation or are reduced, through an enzymatic reaction, to beta-hydroxybuterate (3HBA), in order to generate energy for the mammal's body. Glycerol is converted into sugar via gluconeogenesis. 3HBA can be transported from the liver to other

tissues and be converted to acetyl-CoA to produce energy (Bezerra et al. 2014; Currie 1992; Campbell & Farrell 2012).

1.3.7.3. Non-esterified fatty acids

The increase of non-esterified fatty acids concentrations in blood during the transition period seem to be connected to the onset of the metabolic disorders ketosis, hepatic lipidosis, and milk fever. This is the result of negative energy balance due to high energy mobilisation. (Bezerra, et al., 2014). Throughout the negative energy balance period, there is an alteration of tissue responsiveness and key hormone expression that cause an increase of lipolysis and decrease of lipogenesis. As a result of this, non-esterified fatty acids and 3HBA concentrations are high. These high concentrations indicate fatty acid oxidation and lipid mobilisation. Ketosis occurs when there is an imbalance in fat metabolism and hepatic carbohydrate metabolism due to excessive fat mobilisation. In other words, when there is a negative energy balance, fatty acid oxidation and ketone body production occurs (Bezerra, et al., 2014). Fatty liver occurs when plasma contains an elevated level of non-esterified fatty acids. The ability of the liver to take up non-esterified fatty acids is proportional to non-esterified fatty acid concentration in the blood. When non-esterified fatty acids are taken up by the liver, one of two things can occur: esterification to triglycerides, or oxidation in mitochondria or peroxisomes. When insulin and glucose levels in the blood are low, there is an increase in the liver uptake of non-esterified fatty acids (Bezerra, et al., 2014).

Furthermore, when non-esterified fatty acids are completely oxidized, CO_2 is formed, while ketone bodies are formed when there is an incomplete oxidation. Measuring ketone bodies in serum and ketones in urine can assist in confirming the diagnosis of fatty liver syndrome (Bezerra, et al., 2014).

1.3.8. Foetal-Neonatal nutrition

There is a relationship between diet and metabolism. Metabolites can be generated from endogenous metabolic processes or exogenous dietary nutrients. Analysing metabolites enables diagnosis of diseases and disorders at an early stage. It could also be useful to determine new predictive markers. Epidemiological studies involving animals and humans have shown that, when there are metabolic disturbances and nutritional imbalances during the important times of foetal development during pregnancy, there may be persistent effects on the health of the neonates and later in adulthood (Bagchi et al. 2015). Nutrient imbalance and foetal malnutrition during pregnancy may cause the development of metabolic disorders

such as metabolic syndrome in postnatal life (Bagchi et al., 2015; Castrogiovanni & Imbesi, 2017).

1.3.9. Nutrimetabolomics

Metabolomics is a useful tool in nutrition research. Nutrimetabolomics involves the study of the metabolome in terms of nutritional status or challenge concerning humans or animals.

Foetal growth restriction as in intrauterine growth restriction (IUGR) can be caused by energy metabolism and disorder of nutrients. Analysis of umbilical vein plasma showed a marked difference in the concentration of metabolites when IUGR foetal pigs were compared with normal birth weight foetal pigs (Bagchi, et al., 2015).

Maternal diet, among other factors, can influence foetal growth. Insufficient nutrients can affect metabolism and cause metabolic disturbances. Sufficient nutritional substrates provided across the placenta are required to prevent foetal growth retardation (Bagchi, et al., 2015).

There are high foetal demands for glucose and the placenta transports glucose from maternal plasma to foetal plasma. An imbalance between foetal consumption of glucose and maternal synthesis and absorption of glucose can result in maternal hypoglycaemia. Lipolysis then occurs and long chain fatty acids (LCFA) are released from adipose tissue which are then taken up by the liver. This will result in ketosis (Kaneko et al., 2008) where negative energy balance occurs due to energy consumption being too low to support the high energy demands during the transition period.

Ketosis during lactation is more complex than ketosis under foetal energy demands. Glucose is the precursor of lactose. During lactation there is a drain on plasma glucose. In ruminants, most of the plasma glucose is synthesized by gluconeogenesis in the liver. Propionate and amino acids are the main substrates. The other precursors are glycerol, lactate, acetate, and buyrate. When there is not enough glucose produced via gluconeogenesis to match the requirements for lactose synthesis, hypoglycaemia will occur and then lead to ketosis (Kaneko, et al., 2008).

1.4. Serum metabolic studies in cows

This section reviews the metabolites reported in the blood of cows.

1.4.1. Cow

In a study on Holstein dairy cows, it was suggested that there is a relationship between blood metabolites and the resumption of postpartum cyclicity. Increased serum non-esterified fatty acids, 3-hydroxybutyric acid (3HBA), albumin, urea nitrogen, total cholesterol, and magnesium are linked to negative energy balance in the postpartum period which is associated with delayed resumption of postpartum cyclicity (Jeong et al. 2015).

In another study on Holstein dairy cows, serum γ-glutamyltransferase and total cholesterol showed no changes in ketogenic and non-ketogenic groups. During the postpartum period, a higher amount of serum non-esterified fatty acids, a higher amount of aspartate aminotransferase, a lower amount of urea nitrogen, and a lower amount of glucose, were reported for a ketogenic group of dairy cows when compared with a non-ketogenic group. It was concluded that a negative energy balance during early postpartum was associated with ketosis (Shin et al. 2015).

In a study on ketone bodies in Holstein cow milk, ketone bodies were detected in cow blood and milk. Acetoacetate, 3HBA and acetone are ketone bodies and metabolites of fatty acid oxidation which will appear in increasing amounts in milk and tissues. Blood acetone, acetoacetate, and 3HBA have been detected in cows. Cow milk acetoacetate and 3HBA were present at lower concentrations when compared to cow blood (Enjalbert et al. 2001).

In a study on Holstein Frisian cows, parity and postpartum intervals significantly affected serum glucose concentration, serum cholesterol concentration, and serum cortisol concentration. Parity does not significantly affect serum triglyceride concentration, whereas postpartum intervals do so significantly. In that study, it was shown that there was a trend of increasing serum glucose during the postpartum period. This is likely because energy for the resumption of reproductive function was provided by glucose. After parturition, the concentration of serum cholesterol steadily increased. There was a trend of increasing serum cholesterol and serum triglycerides during the postpartum period. The results also showed that high-yielding cows were more prone to negative energy balance (Najmus et al. 2018).

Energy status of cows in early lactation can be indicated by free fatty acid levels in the blood, more so than glucose; during early lactation there is excessive variability of both which limits its usefulness (Erfle et al. 1974; Adewuyi & Gruys 2011). Blood glucose was inversely correlated with blood acetoacetate and 3HBA in a study on the interrelationships between blood metaboilites (Erfle et al. 1974).

1.4.2. Pregnant dairy cow

In a study characterizing the maternal plasma metabolic response to successful pregnancy in pregnant Holstein cows, shifts in concentrations of metabolites were reported. The three time points selected in this study were day 0 (conception), day 17(embryonic phase), and day 45 (foetal phase). Plasma glycerophospholipid metabolites, including triacylglycerols, diacylglycerols, and glycerol 3-phosphate, decreased on day 17 and 45 of pregnancy. It was suggested that this is associated with alterations in the frequency of use of fatty acids in β-oxidation for energy production, which is required by the uterus for the embryo and foetus. Plasma alpha-linolenic acid levels decreased on these same days and it may be because long chain poly-unsaturated fatty acids are utilised by the mother for embryo and foetal development. Folate biosynthesis metabolites such as folic acid and tetrahydrofolic acid also showed a decrease, possibly due to folic acid transportation to the uterus for embryo and foetal development. Plasma pantothenic acid also decreased on day 45. Metabolites associated with nucleic acid biosynthesis, including metabolites from nucleotide sugar metabolism and amino sugar metabolism, such as N-Acetyl-D-glucosamine and fucose, decreased on day 45. These metabolites may diminish in maternal plasma to generate energy for the uterus without the use glucogenic precursors (Guo & Tao 2018).

1.5. Interspecies comparison of milk metabolites

This section reviews the metabolites reported in the milk of different species. There are metabolites which are present in the milk of one species but not in that of another. In section 1.3 and 1.4, literature suggested that metabolites that are involved in major metabolic pathways, such as glycerol, should be detectable in the milk of all species. Fatty acids are synthesized in the mammary gland (Palmquist, 2006) and this synthesis requires glycerol to be available (Frandson et al., 2009). A comparison of metabolites between species (See Tables A1 – A6 in appendix for details) may suggest that either some metabolites, such as glycerol, are not present in the milk of certain species, or that due to shortcomings in methods of analysis these metabolites were not detectable.

In the study on Holstein cows, gas chromatography–mass spectrometry (GC-MS) analyses were employed, and only part of the milk metabolome was measured. Predominantly short-chain water-soluble metabolites, involved in energy metabolism, were detected (Melzer et al., 2013). Therefore, some metabolites seemed to be absent in Holstein cow's milk, when compared to the study done on the Brown Swiss and Simmental cows, which used Nuclear magnetic resonance (NMR) (Klein et al. 2010).

The shortcomings of the different analytical methods used to analyse the metabolites complicates comparison. NMR spectroscopy may not require additional steps for sample preparation, however, MS-based metabolomics provides an increased sensitivity. When compared to NMR, MS sample preparation is more demanding and will need different columns and requires the optimizing of ionization conditions. NMR can detect all metabolites at the detectable concentration level in one measurement, however, MS requires different chromatography techniques for different groups of metabolites. The sensitivity of MS is higher than that of NMR and may be able to detect metabolites below the detection level of NMR (Emwas, 2015).

There is no literature found on the milk metabolomics of wild mammals. Tables of comparison between species for serum metabolites were not possible to compose due to the limited amount of information available. The following sections discusses the metabolites in the milk of different species.

1.5.1. Metabolome of cow's milk

The health status of a cow is related to the biochemical profile of their milk. Certain compounds are highly correlated to the metabolic status of the individual animal. Klein et al. (2010) quantified 44 metabolites in milk of dairy cows during early and late lactation. It was shown that citrate is positively correlated with ketones, de novo fatty acid synthesis, and 3HBA in milk. Milk acetone and milk 3HBA are energy status markers in the cow because concentrations above the threshold detection levels will indicate subclinical ketosis (Konar, A. & Rook. 1971; Klein et al. 2010).

In early lactation, choline was present in lower values and phosphocholine was present in higher values when compared to the last lactation third. Choline is obtained from maternal circulation uptake and de novo synthesis within the mammary gland. There is a positive correlation between free choline in milk and milk protein content because choline is a part of the metabolic pathways of methyl groups (Klein et al. 2010).

Ornithine, a precursor of several nonessential amino acids and proline, was shown to be varied between the two breeds of cows (Brown Swiss and Simmental). Lactose was shown to remain constant between the two breeds (Klein et al. 2010).

Talose and malic acid are metabolites which have been noted as unique to bovine milk (Scano et al. 2014). Creatinine content is higher in buffalo milk when compared to cow milk (Park &

Haenlein 2013). 2-Piperidinecarboxylic acid is regarded as an important metabolite for casein content in Holstein cow milk and was negatively correlated to casein (Melzer et al. 2013).

1.5.2. Metabolome of goat's milk

Goat milk is notably rich in branched chain fatty acids. Valine and isoleucine are present in goat milk and they are intermediates in branched chain fatty acid synthesis (Massart-Leën & Massart 1981; Scano et al. 2014). Ribose was reported to be a discriminant metabolite when compared to cow milk (Scano et al. 2014).

1.5.3. Metabolome of giant panda milk

In contrast to panda milk, bovine milk contains a high level of lactose and low levels of oligosaccharides. Isoglobotriose is an oligosaccharide and was one of the 50 metabolites identified in Giant panda milk. The abundance of this trisaccharide changed over time for three pandas under the study (Zhang et al. 2015). Isoglobotriose has been noted to be a major oligosaccharide in the milk of the Ursidae family (Urashima et al. 2013; Zhang et al. 2015). There was an initial peak of lactose and isoglobotriose in Giant panda milk, but after 18 days these fell to a plateau (Zhang et al. 2015).

The lactose content in panda milk, as well as many members of the Carnivora order, has been reported to be relatively low in comparison to species of the Bovidae family as well as humans (Xuanzhen et al. 2005; Zhang et al. 2015; Holt & Carver 2012; Oftedal 2012; Langer 2009; Nakamura et al. 2003).

Two disaccharide isomers confirmed as 3'-sialyllactose and 6'-sialyllactose have been identified in Giant panda milk and have been reportedly found in bovine milk (Zhang et al. 2015; Kelly et al. 2013). Gc2-3Lac is a sialy-lated disaccharide of Giant panda milk (Zhang et al. 2015). Sialyated oligosaccharides have been noted to have potential benefits related to immune function, gut microbiome maturation, and promoting resistance to pathogens (ten Bruggencate et al. 2014; Zivkovic & Barile 2011; Lane et al. 2012; Weiss & Hennet 2012). The components identified in giant panda milk also include amino acids such as beta-Citryl-L-glutamic acid which are not present in the milk of other species as shown in Table A2. Linoleic acid and eicosenoic acid are two of the fatty acids also identified in giant panda milk which were not detected in other species (Table A5) (Zhang et al. 2015).

1.5.4. Metabolome of donkey milk

A change in the metabolite profile of donkey milk over lactation has been reported. As lactation progressed, there was an increase in the content of protein, urea, glyceric acid, pyroglutamic acid, and aspartic acid. There was a decrease in malic acid, uracil, talose, phosphate, and and myo-inositol. The donkey milk metabolite profile was shown to be more like human milk when compared to cow milk (Murgia et al. 2016). Amino acid related metabolites seem to be the most abundant group of metabolites present in donkey milk when compared to other species as shown in Table A2. Donkey milk contains no vitamin related metabolites in comparison to other species as shown in Table A6.

1.5.5. Metabolome of human milk

The non-essential amino acids taurine, alanine, glutamine, and glutamate occurred most abundantly. Fucose is a notable metabolite found in human milk because it is the core monosaccharide of fucosylated oligosaccharides and has been thought to be the product of glycosidase reactions on oligosaccharides. The variation in the human milk metabolome has been associated with diet alterations (Smilowitz et al. 2013).

1.6. Nutrient composition of milk

This section summarizes the composition of milk and gives an overview of the synthesis of milk nutrients. The milk nutrient concentrations of different species are shown in Table 1.1.

1.6.1. Carbohydrates

A carbohydrate is a molecule consisting of carbon, oxygen and hydrogen atoms. Carbohydrates are largely classified as monosaccharides, disaccharides, or polysaccharides. Monosaccharides are the simple sugars. Disaccharides are composed of two monosaccharides. Polysaccharides are composed of many monosaccharides linked together. Carbohydrate molecules consisting of up to ten monosaccharides are known as oligosaccharides (Li & Khanal, 2017; Campbell & Farrell, 2012).

Carbohydrates are found in milk as well as plant sources such as grains and vegetables (Li & Khanal, 2017). Carbohydrates in milk are the main source of energy for the calf (Webster, 2019). Oligosaccharides in milk are important for the calf as they function as prebiotics and are important for maintaining gut health (Zivkovic & Barile, 2011). Factors such as stage of

lactation (Henao-Velásquez et al., 2014), pregnancy (Gurmessa & Melaku, 2012) and diet (Grainger et al., 2009; Mosavil et al., 2012) can influence the carbohydrate content of milk.

α-lactalbumin and galactosyltransferase make up the lactose synthase system and they are responsible for the synthesis of lactose (Hettinga, 2019). Lactose is synthesized inside the secretory cells of the mammary gland. These cells synthesize galactose by using glucose from the blood. The galactose is then combined with glucose to form lactose. Blood glucose in ruminants is primarily obtained from gluconeogenesis in the liver. The liver makes use of the substrate propionic acid, a volatile fatty acid, which is absorbed from the rumen. Because very little glucose is absorbed from the gastrointestinal tract, blood glucose in ruminants is low in comparison to other mammals (Frandson et al., 2009). Oligosaccharides may be synthesized within the mammary glands due to the action of several glycosyltransferases making use of lactose as an acceptor (Pontarotti, 2014).

1.6.2. Fat and fatty acids

A fatty acid is a carboxylic acid that consists of a hydrocarbon chain as well as a terminal carboxyl group. Fatty acids differ by length of the hydrocarbon chain and the chain can either be saturated or unsaturated (Bajpai, 2014; Campbell & Farrell, 2012). Saturated fatty acids have no carbon-carbon double bonds. Unsaturated fatty acids have one or more carbon-carbon double bonds. Triglycerides are the major form of fat and are composed of three fatty acids and a glycerol backbone (Bayly, 2014).

Essential fatty acids, such as linoleic acid, cannot be synthesized by the body and need to be obtained from the diet (Kapalka & Kapalka, 2010). Milk fat provides energy for the calf and is important for growth and for its immune system (Palmquist, 2006; Hill et al., 2011; Miller, 1979). Diet (Woods & Fearon, 2009; Chilliard et al., 2001), stage of lactation (Nantapo et al., 2014), genetic parameters and animal individuality, (Hanuš et al., 2018) significantly affect the content of fatty acids in milk.

Fatty acids in milk are derived directly from the blood and from biosynthesis in the mammary gland (Palmquist, 2006). Butyric acid is also derived from rumen bacteria. Rumen bacteria also produces acetate which can be linked to butyric acid by covalent bonds to from medium chain fatty acids (Barbosa-Cánovas et al., 2006; Christie, 2014).

Short chain fatty acids are synthesized in high quantities. The two kinds of enzymes responsible for milk fatty acid biosynthesis are chain elongating enzymes and chain terminating enzymes. The fatty acid synthase complex, found in mammary gland cells,

contains fatty acid synthases which synthesize fatty acids to a required length (Christie, 2014). Ruminants possess a fatty acid synthase with broad acyl chain-length specificity. This enzyme produces short chain acyl-CoAs which are utilized for triglyceride synthesis in the mammary gland (Vance & Vance, 2008). A mammary gland specific thioesterase terminates chain elongation by cleavage (Christie, 2014).

Glycerol is derived primarily from the catabolism of glucose through glycolysis. Blood 3HBA and acetate provide carbon required for fatty acid synthesis. Acetate is the primary source of carbon. These two molecules are produced as volatile fatty acids by fermentative metabolism by rumen microorganisms. The volatile fatty acids are absorbed into the blood from the rumen and become available to the mammary gland (Frandson et al., 2009).

1.6.3. Proteins

A protein is a large molecule composed of one or more chains of linked amino acids. Amino acids are molecules which contain a carboxylic acid group, an amine group, and a side-chain (Sparkman et al., 2011). Amino acids are categorized according to their side chains. The side chains my either be polar or nonpolar (Campbell & Farrell, 2012).

Protein can be found in animal products such as meat and milk. Proteins are also in plants, at lower amounts. There are eight essential amino acids which are not able to be synthesized by the body and need to be obtained from the diet (Litwack & Litwack, 2018). Milk protein is important for growth, development and immune function of young animals (Li et al., 2007; Barbosa-Cánovas et al., 2006). The protein portion in milk is the most variable non-fat milk component and can be influenced by factors such as stage of lactation. The protein content decreases after colostrum milk and begins to increase again towards the end of lactation. Diet is also an influencing factor and a diet deficient of protein can lower the milk protein content. Environmental factors such as age can also cause the milk protein content to slowly decline over time (Owen et al., 1984).

A combination of local and systemic mechanisms is responsible for the synthesis of milk proteins (Rius et al., 2010). These mechanisms are not well understood (Dan et al., 2016). Mammary gland secretory cells directly synthesize casein proteins using amino acids from the blood. The mammary glands also synthesize the whey proteins alpha-lactalbumin and beta-lactoglobulins. (Frandson et al., 2009). Once inside the mammary gland cell, amino acids are covalently bound at the rough endoplasmic reticulum to form proteins. These proteins are transported to the Golgi apparatus where they can undergo post-translational processes and be excreted. The proteins are transported to the apical membrane through secretory vesicles.

The vesicle contents are then discharged into the alveolar lumen and into the milk (Fox et al., 2015; Fox & Mcsweeney, 1998). Immunoglobulins, which are also categorised as whey proteins, are produced by lymphocytes and the liver produces serum albumin. These whey proteins are transported through the blood to the mammary glands from where they are excreted in the milk (Frandson et al., 2009).

1.6.4. Interspecies comparison of milk nutrients

The same major nutrients – protein, fat, and carbohydrates – are present in the milk of all mammals. However, the amounts of these nutrients may differ between species (Kon & Cowie, 2016) and taxonomic orders (Osthoff et al. 2017). Examples are the high fat content of marine mammal milk (Oftedal et al., 2014; Oftedal et al., 1995). Members of the Alcelaphinae family, such as the wildebeest (*Connochaetes*), have been shown to have a high content of medium chain fatty acids in the milk (Osthoff et al., 2009; Osthoff et al., 2017). A high oligosaccharide content of elephant (*Loxodonta africana*) milk (Osthoff et al., 2005) has also been reported.

The milk nutrient composition of representatives from different orders, cow (*Bos Taurus*), goat (*Capra aegagrus hircus*), giraffe (*Giraffa*) of the order Artiodactyla, donkey (*Equus asinus*) of the order Peryssodactyla, and giant panda (*Ailuropoda melanoleuca*), and cheetah (*Acinonyx jubatus*) of the order Carnivora are shown in table 1.1. The nutrient concentration differences between species and factors, such as nutrition, can affect the quality of milk and its components (Mackle et al., 1999; Jenkins & McGuire, 2006; Tyasi et al., 2015).

Protein makes up 95% of the nitrogen content in milk. The remaining 5% is NPN (non-protein nitrogen). Urea makes up the largest contribution to the NPN fraction (Walstra, 1999; Barbosa-Cánovas et al., 2006) and is derived from amino acid catabolism (Campbell & Farrell, 2012). Whey and casein are the two major proteins. Milk also contains other proteins such as enzymes and membrane proteins (Walstra, 1999; Barbosa-Cánovas et al., 2006). As seen in Table 1.7, the greatest difference in milk nutrient composition between herbivores and carnivores is the high protein content in carnivore milk (Skibiel et al., 2013). Carnivore milk contains a higher protein content when compared to ruminants in Table 1.1 and this is because carnivores have a higher protein requirement (Ewer, 1998).

Around 98% of milk fat consists of a variety of triglycerides. Seventy percent of milk fat is unsaturated with the most abundant fatty acid being oleic acid. Fatty acids with 4 to 18 carbons are most abundant in milk. Short chain fatty acids (C2-C6) make up a significant proportion of the milk fat of milk from ruminants (Walstra, 1999; Barbosa-Cánovas et al., 2006). Short chain fatty acids are present in lower amounts in the milk of non-herbivores (giant panda) and

carnivores (cheetah) as shown in Table 1.1. Milk of ruminants have a higher amount of these short chain fatty acids as they are produced by the rumen bacteria (Chesworth et al., 1998; Christie, 2014). Although the giraffe is a ruminant, its milk contains a higher amount of fat when compared to the two common domestic ruminants. Fat content as well as fatty acid content can differ between orders and individual species within an order, which is an evolutionary development of nutrient requirements of the offspring (Oftedal et al., 1995; Oftedal, 2012; Berdanier et al., 2007)

The main carbohydrate in milk is the disaccharide lactose. Traces of glucose are also present (Mahindru, 2009; Walstra, 1999). Oligosaccharides, which are also present in milk in small amounts, function as prebiotics (Zivkovic & Barile, 2011). Oligosaccharides are not present in the milk of most of the species listed in Table 1.1 because they normally occur in small amounts and are difficult to detect (Yan et al., 2017). The lactose content in milk of many members of the Carnivora order has been reported to be relatively low in comparison to ruminants and herbivores (Holt & Carver 2012; Oftedal 2012; Zhang et al. 2015), however, as shown in Table 1.1, cheetah milk seems to be an exception. Lactose content in the milk of different species can vary widely (Zadow, 1992).

Table 1.1: Content of macronutrients and selected fatty acids in the milk of mammals representative of different taxonomic orders

Order	*Bos Taurus*	*Capra aegagrus hircus*	*Giraffa*	*Equus asinus*	*Ailuropoda melanoleuca*	*Acinonyx jubatus*
Nutrient (g/100g milk)	Cow[1, 2, 4]	Goat [2, 3,]	Giraffe[1]	Donkey[5, 6, 7]	Giant panda[8]	Cheetah[9]
Total protein	3.27[1]	3.4[2]	4.9	1.63[5]	7.75	9.96
Whey	0.63[1]	0.6[3]	1.77	0.64[5]	-	6.53
Casein	2.6[1]	2.11[3]	3.14	0.7[5]	-	3.42
Fat	3.9[1]	3.8[2]	7.94	0.5-1.7[6]	11.17	6.48
Butyric acid (C4:0)	2.87[4]	2.03[4]	0.65	0.57[6]	0.46	-
Caproic acid (C6:0)	2.01[4]	2.78[4]	1.75	1.16[6]	0.36	-
Caprylic acid (C8:0)	1.39[4]	2.92[4]	2.67	2.33[6]	0.13	-
Capric acid (C10:0)	3.03[4]	9.59[4]	8.56	6.58[6]	0.13	-
Lauric acid (C12:0)	3.64[4]	4.52[4]	1.28	6.99[6]	0.3	0.8
Oleic acid (C18:1c9)	22.36[4]	18.6[4]	16.91	17.0[6]	21.86	32.4
Lactose	4.8[1]	4.1[2]	4.16	7.0	0.82	4.02
Galactose	0.05[1]	-	-	-	-	0.1
Oligosaccharides	-	-	-	-	-	0.2

Ash	0.70[1]	0.86[2]	-	-	0.92	-
Calcium (mg/100g)	122[2]	134[2]	-	807.09[7]	207.67	-

[1](Osthoff et al., 2017); [2](Park, 2016); [3](Kumar et al., 2012); [4](Markiewicz-Keszycka et al., 2013); [5](Martini et al., 2017); [6](Gastaldi et al., 2010); [7](Fantuz et al., 2012); [8](Zhang et al., 2016); [9](Osthoff et al., 2006)

1.7. Research on milk of wild mammals

There is little information available on the milk of wild mammals. Unique characteristics of milk in general may be discovered in milk of animals other than the domesticated species. The milk composition of the Alcelaphinae subfamily contains a high content of medium chain saturated fatty acids (Osthoff et al. 2009; Osthoff et al. 2012; Osthoff et al. 2017). Milk of the African elephant contains a high content of oligosaccharides together with lactose (Osthoff et al. 2005; Osthoff et al. 2008), and also is devoid of α-casein (Madende et al. 2015). The milk of seals contains trace amounts lactose, due to the absence of α-lactalbumin (Reich & Arnould, 2007; Osthoff, 2016). These findings have brought about an interest in further investigation of milk from other wild mammals.

1.8. Conclusion

Milk of all mammals consist of similar major nutrients. However, the amounts of these nutrients may differ between species (Kon & Cowie, 2016). Certain factors such as diet and stage of lactation can cause alterations in the concentrations of these major milk nutrients (Owen et al., 1984; Chilliard et al., 2001; Rego et al., 2016).

Because a large amount of blood is required to pass through the udder and the components in the blood are used to synthesize milk nutrients (Walstra et al. 2005; Mansour et al. 2017), other blood components unrelated to milk synthesis, such as rumen microbe related metabolites (Hungate, 1966; Lees et al. 2013), could end up in the milk. For this same reason, blood metabolites can be related to milk metabolites and milk nutrients.

The milk metabolome differs between species (Klein et al., 2010; Melzer et al., 2013) and this is shown in Tables A1 to A6 in appendix A. There is even a difference between the metabolites in Holstein cow milk and Brown Swiss and Simmental cow milk, although the possibility of differences due to different analytical methods used, cannot be ruled out (Emwas, 2015). The

human milk metabolome seems to be most abundant in saccharides and amino acid related metabolites. Goat milk seems to be abundant in saccharides and sugar alcohol related metabolites as shown in Table A3. Giant panda milk seems to be abundant in amino acid related metabolites as shown in Table A2 and donkey milk is not particularly abundant in any category of metabolites. Currently there is very little information available on the milk metabolome of different species and there is no information available whether the blood metabolome may have an effect on the milk metabolome and/or the milk nutrients.

Inter-species comparison of milk of Monotremata, Marsupialia and Eutheria has lead to an understanding of the evolution of many aspects of milk. The most far reaching were the evolutionary development of the caseins (Rijnkels, 2002; Lefevre et al. 2009) and α-lactalbumin (Prager and Wilson, 1988; Qasba and Kumar, 1997), which in turn was responsible for the high content of lactose in milk (Hoppe and McKenzie, 1974; Oftedal et al. 1987). The field of milk metabolomics is therefore open for investigation and comparisson. It would be of specific interest to find out whether an interrelation between metabolomes and milk nutrients exists, specifically in the mammals with unique milk characteristics. Since the giraffe is the largest ruminant, and its milk composition differs from other ruminant taxa (Osthoff et al. 2017) it would be of interest to compare its metabolome with that of others, such as the cow and goat.

1.9. Aims

A group of giraffes, including lactating females, were available for research of milk-related aspects. The aims of this study were:

- to obtain a baseline metabolome of giraffe serum
- to obtain a baseline metabolome of giraffe milk
- to determine whether there is any significant interrelationship between the metabolomes and milk nutrients

Chapter 2 - Metabolites in giraffe serum

2.1. Introduction

The metabolome of a mammal consists of a large number of molecules of which each is within a concentration range, indicating a baseline of the metabolome. The metabolites are breakdown products of nutrients, as well as building blocks for new molecules in the cells (Bagchi et al., 2015; Lämmerhofer & Weckwerth, 2013). Milk cells are specialized cells which, apart from maintaining their own existence, also use the blood metabolites to synthesize nutrients that are eventually secreted in the milk (Frandson et al., 2009). Fatty acids, glycerol, 3HBA, and acetate are used to synthesize milk fat. Fatty acids in milk have two sources; derived directly from the blood, as well as de novo biosynthesis in the mammary gland (Palmquist, 2006).

Blood glucose in cattle is predominantly obtained from gluconeogenesis in the liver. In ruminants, the liver makes use of propionic acid obtained from the rumen to produce glucose (Yost et al., 1977). The blood glucose is used by the mammary glands to first synthesize galactose, which is then combined with glucose to form lactose. The mammary gland secretory cells also synthesize milk proteins using amino acids from the blood (Frandson et al., 2009). One of the proteins synthesized is α-lactalbumin, which in turn is key in the synthesis of lactose (Melzer et al., 2013; Brew et al., 1968).

Metabolites in giraffe serum have not yet been reported. In the current study the opportunity was taken to simultaneously study the metabolome of serum and milk, as well as the milk macronutrients. The study subjects consisted of male and female giraffes. The females were either dry, nursing or pregnant. Some were pregnant while nursing a previous offspring. Two females were from a different location. In this chapter, the metabolome of giraffe serum was analysed to obtain a baseline of metabolites.

2.2. Materials and methods

2.2.1. Study site and sample collection

This research forms part of multi-disciplinary research on giraffes, where giraffes were darted to fit radio transmitter collars. Ethical approval was obtained from the Animals Research Ethics of the University of the Free State (UFS-AED2016/0106). The study site was the Rooipoort Nature Reserve near Kimberley in the Northern Cape. Giraffes were sedated and fitted with radio transmitters during early summer of one year (2017), and transmitter removal was carried out a year later (2018). Blood and milk was collected during these operations. The animals were free roaming and browsed on natural vegetation. No supplementary fodder was made available to the animals. The differences in the rainfall received are described in Appendix B, and it was assumed that it affected the availability of nutrients for the giraffes. Blood and milk were also collected from two giraffes that were culled in the Sandveld Nature Reserve, Hoopstad, Free State Province. Blood obtained from giraffes at Rooipoort Nature Reserve were numbered F, taken in 2017 and BF in 2018. Samples from Sandveld Nature Reserve were labelled SV (Table B1 in appendix B).

The stage of lactation ranged between 2.5 and 4.5 months postpartum. Compared to cows, this falls in mid lactation. Blood (4 ml) was drawn with an evacuated tube (BD Vacutainer ® K3E, 7.2 mg) from the jugular vein. The blood was kept on ice while in the field, and the serum cleared by centrifugation (Hettich, EBA 12; 800 rpm, 5 minutes) within 3 hours. Serum was stored frozen at -20°C until analysed.

2.2.2. Sample preparation for NMR analysis

The blood samples were filtered using Amicon Ultra – 2 mL centrifugal units with 10kDa membrane filters (Merck; Ref UFC201024). Each centrifugal unit was pre-rinsed twice using 2 mL dH_20 (double distilled water) at 4500 g for 15 minutes in a swing-bucket centrifuge. The rinsing was done to remove trace amounts of glycerol from membrane filters, which can interfere with NMR signals. One mililiter of serum was placed in a microcentrifuge tube and centrifuged at 12000 g for 5 minutes, and 600 µl serum was then filtered by centrifugation at 4500 g for 30 minutes in a swing-bucket centrifuge using the membrane filters. To 540 µl of filtered serum, 60 µl NMR buffer solution (1,5 M potassium phosphate solution in deuterium oxide with internal standard TSP (trimethylsily1-2,2,3,3-tetradeuteropropionic acid); pH 7.4)

was added. The sample was mixed by vortex to ensure that it is completely homogenous, and then transferred to a 5 mm NMR tube for analysis.

2.2.3. ^{1}H-NMR analysis

The prepared samples were subjected to NMR spectroscopy on a Bruker Avance III HD NMR spectrometer, equipped with a triple resonance inverse (TXI) 1H{15N, 13C} probe head and x, y, z gradient coils, at 500MHz. ^{1}H spectra were acquired as 128 transients in 32K data points with a spectral width of 6002 Hz and acquisition time of 2.72 sec. Receiver gain was set to 64. The sample temperature was maintained at 300K and the H_2O resonance was presaturated by single-frequency irradiation during a relaxation delay of 4s, with a 90° excitation pulse of 8µs. Shimming of the sample was performed automatically on the deuterium signal. Fourier transformation and phase and baseline correction were done automatically. Software used for NMR processing was Bruker Topspin (V3.5). NMR spectral analysis, peak annotation and quantification was done using Bruker AMIX (V3.9.14) (Ellinger et al. 2013).

2.2.4. Statistical analysis

Statistical analysis of all the results was carried out with JMP Statistical Software (JMP® Software, 2019). Principal component analysis (PCA) plots were used to identify separation between the groups of giraffes. Pre-processing of the data was done by excluding all metabolites which had multiple zero values for the animals. A non-parametric method of analysis was chosen because of the small giraffe sample size. Significant differences were noted at $p < 0.05$.

2.2.5. MetaboAnalyst pathway analysis

The online MetaboAnalyst software (www.metaboanalyst.ca) was used to detect the pathways of the metabolites which were significantly different between the two years. The pathway analysis option was selected. A ‘one-column compound list’ was chosen and the data input type was selected to be ‘compound name’. The pathway parameters were set to be a ‘Hypergeometric Test’ for the Over Representation Analysis and ‘Relative-betweeness Centrality’ was selected for the Pathway Topology Analysis. The pathway library selected was ‘Homo sapiens (KEGG) [80]’. A plot was generated showing the pathways of most impact. The large, red dots farthest to the top right corner show the most important pathways. The

light blue compound colour within the pathway indicates those metabolites which are not in the giraffe data and are used as background for enrichment analysis (Chong, Wishart, et al., 2019; Xia & Wishart, 2011b; Xia et al., 2012; Xia et al., 2015; Xia & Wishart, 2016; Chong et al., 2018; Chong & Xia, 2018; Chong, Yamamoto, et al., 2019; Xia & Wishart, 2010; Xia, Wishart, et al., 2011; Xia, Sinelnikov, et al., 2011; Xia et al., 2013; Xia et al., 2009; Xia & Wishart, 2011a).

2.3. Results

An example of ^{1}H-NMR spectra of giraffe serum is shown in Figure 2.1. The mean concentrations of the metabolites (µM) in the serum of giraffes are shown in Table 2.1. Figure 2.2 to 2.6 show the results from the MetaboAnalyst pathway analysis. The PCA plots showed no separation between the groups of giraffes. These plots are labelled B1 to B4 in appendix B. Samples F6 and F8 contained unusually high levels of formic acid and ethanol and lysine and glutamine were below the levels of detection. The high level of ethanol indicated that they were contaminated in some way and that fermentation could have occurred (Jones, 2002; Winek & Esposito, 1985). They were therefore excluded from further analysis.

The significance levels for serum metabolites are shown in Table B2 and B3. The metabolites that differ significantly ($p < 0.05$) with regard to sex (creatinine, pyruvic acid, threonine, citric acid, phenylalanine, and glutamine), were subjected to the MetaboAnalyst pathway analysis, and are shown in Figures 2.2 and 2.3. The figures show that the alanine, aspartate, and glutamate metabolism, as well as the citric acid cycle, were more active in male giraffes as the concentrations of these metabolites are higher than in the serum of the female giraffes as shown in table 2.1. The metabolic demands of the female giraffes were greater due to lactation and pregnancy, and a higher activity of these pathways was expected for the male giraffes.

The metabolites that differ significantly ($p < 0.05$) with regard to year of sampling (citric acid, betaine, creatinine, formic acid, acetone, pyruvic acid, myo-Inositol, glutamine, dimethyl sulfone, allantoin, fumaric acid, histidine, alanine, threonine, mannose, tyrosine, leucine, succinic acid, and phenylalanine) for Rooiport male and females were subjected to the MetaboAnalyst pathway analysis, and are shown in Figures 2.4 and 2.5. The figures show that the alanine, aspartate, and glutamate metabolism, as well as the citric acid cycle, were more active in the 2018 group, as the concentrations of these metabolites are higher for year 2018 when compared to year 2017.

The metabolites that differ significantly ($p < 0.05$) with regard to year of sampling (formic acid, citric acid, and myo-inositol) for Rooiport females only were subjected to the MetaboAnalyst pathway analysis and is shown in Figure 2.6. The glyoxylate and dicarboxylate metabolism was shown to be more active for the 2018 group, as the concentrations of these metabolites are higher for year 2018 when compared to year 2017.

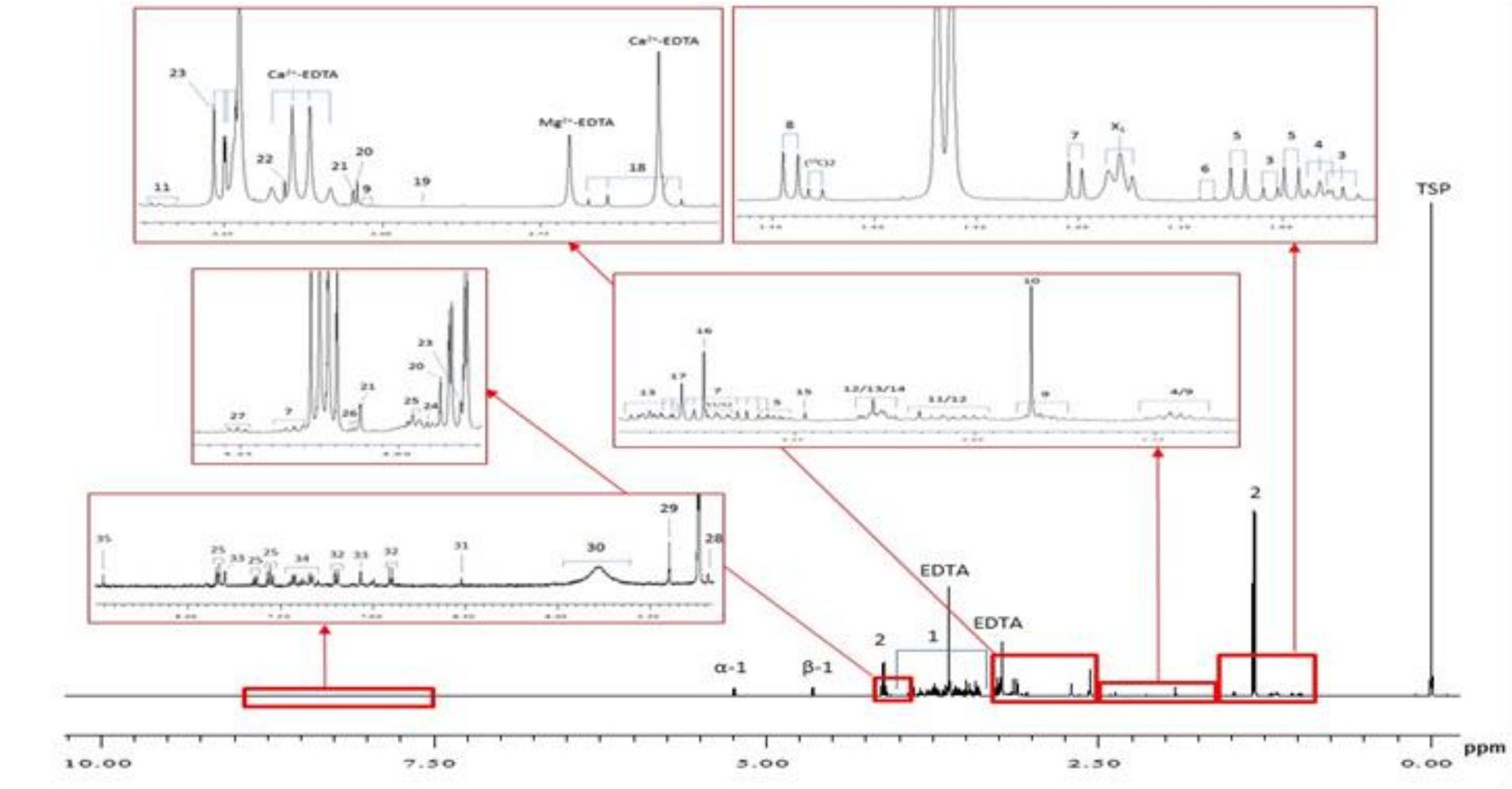


Figure 2.1 = [1]H NMR spectra for giraffe serum. Glucose (3.24 dd, 3.38-3.94, 4.65 d, 5.24 d); 2 = Lactic acid (1.33 d, 4.12 q); 3 = Isoleucine (0.94 t, 1.01 d); 4 = Leucine (0.96 t); 5 = Valine (0.99 d,1.04 d, 2.28 m); 6 = 3-Hydroxyisobutyric acid (1.07 d); 7 = 3-Hydroxybutyric acid (1.20 d, 2.36 AB, 4.16 m); 8 = Alanine (1.48 d); 9 = Lysine (1.73 m, 1.91 m, 3.03 t); 10 = Acetic acid (1.92 s); 11 = Proline (2.01 m, 2.35 m, 3.34 m); 12 = Glutamic acid (2.06 m, 2.13 m, 2.35 m); 13 = Glutamine (2.14 m, 2.45 m); 14 = Methionine (2.14 m); 15 = Acetone (2.23 s); 16 = Pyruvic acid (2.37 s); 17 = Succinic acid (2.40 s); 18 = Citric acid (2.59 AB); 19 = N,N- Dimethylglycine (2.93 s); 20 = Creatine (3.04 s, 3.93 s); 21 = Creatinine (3.045 s, 4.06 s); 22 = Dimethyl sulfone (3.16 s); 23 = Betaine (3.26 s, 3.90 s); 24 = Glycolic acid (3.96 s); 25 = Hippuric acid (3.97 d, 7.56 t, 7.64 tt, 7.84 dd); 26 = myo-Inositol (4.07 t); 27 = Threonine (4.25 ?); 28 = Mannose (5.19 d); 29 = Allantoin (5.40 s); 30 = Urea (5.78 bs); 31 = Fumaric acid (6.52 s); 32 = Tyrosine (6.90 dd, 7.19 dd); 33 = Histidine (7.06 d, 7.80 d); 34 = Phenylalanine (7.38 m); 35 = Formic acid (8.46 s).TSP = trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (0.00 s); EDTA = (3.22 s, 3.62 s); Ca2+-EDTA = (2.56 s, 3.12 q); Mg2+-EDTA = (2.70 s). Not shown: Ethanol (1.18 t, 3.66 q); 3-Hydroxyisovaleric acid (1.25 s)

Table 2.1. Averages of the metabolites (μM) in the serum of giraffes

Metabolite	All giraffes	Females	Non-pregnant females	Pregnant females	Males	Rooiport 2017	Rooiport 2018	Rooiport females 2017	Rooiport females 2018
3-Hydroxybutyric acid	31.77 ± 12.32	36.60 ± 16.12	39.70 ± 12.05	33.49 ± 20.37	26.95 ± 2.96	28.54 ± 9.09	34.97 ± 9.69	28.54 ± 9.09	34.97 ± 9.69
3-Hydroxyisobutyric acid	2.86 ± 0.99	3.25 ± 1.25	3.43 ± 1.53	3.07 ± 1.03	2.48 ± 0.43	2.67 ± 0.63	2.87 ± 0.58	2.7 ± 0.63	2.9 ± 0.58
Acetic acid	33.06 ± 24.88	42.90 ± 31.58	44.56 ± 21.24	41.24 ± 42.26	23.23 ± 9.72	28.53 ± 15.44	34.99 ± 13.46	28.53 ± 15.44	34.99 ± 13.46
Acetone	0.99 ± 0.58	0.80 ± 0.35	0.87 ± 0.44	0.74 ± 0.25	1.18 ± 0.72	0.61 ± 0.14	0.85 ± 0.21	0.6 ± 0.14	0.8 ± 0.21
Alanine	124.11 ± 32.71	109.43 ± 34.28	130.93 ± 33.35	87.92 ± 19.39	138.79 ± 24.60	100.44 ± 38.34	130.35 ± 24.80	100.4 ± 38.34	130.3 ± 24.80
Allantoin	16.71 ± 3.70	15.29 ± 4.34	16.44 ± 5.03	14.13 ± 3.71	18.14 ± 2.36	12.42 ± 2.31	18.44 ± 3.34	12.4 ± 2.31	18.4 ± 3.34
alpha-Glucose	285.61 ± 123.58	259.24 ± 121.65	311.56 ± 146.45	206.91 ± 70.74	311.98 ± 126.09	231.54 ± 101.77	385.78 ± 170.21	231.5 ± 101.77	385.8 ± 170.21
beta-Glucose	218.94 ± 101.24	201.70 ± 100.72	243.98 ± 123.55	159.43 ± 55.63	236.18 ± 104.07	183.07 ± 91.61	296.01 ± 135.60	183.1 ± 91.61	296.0 ± 135.60
Betaine	21.00 ± 5.66	19.25 ± 7.10	21.28 ± 5.95	17.22 ± 8.23	22.76 ± 3.23	15.16 ± 3.77	19.86 ± 3.15	15.2 ± 3.77	19.9 ± 3.15
Citric acid	28.39 ± 11.98	22.35 ± 10.32	24.44 ± 12.92	20.26 ± 7.84	34.43 ± 10.75	15.55 ± 4.17	27.90 ± 5.66	15.5 ± 4.17	27.9 ± 5.66
Creatine	21.52 ± 7.49	21.95 ± 10.14	25.11 ± 10.62	18.79 ± 9.67	21.08 ± 3.89	18.42 ± 9.16	18.41 ± 0.54	18.4 ± 9.16	18.4 ± 0.54
Creatinine	17.90 ± 4.78	15.20 ± 4.59	16.79 ± 5.38	13.61 ± 3.49	20.60 ± 3.31	12.47 ± 3.10	18.82 ± 4.28	12.5 ± 3.10	18.8 ± 4.28

Metabolite	All giraffes	Females	Non-pregnant females	Pregnant females	Males	Rooiport 2017	Rooiport 2018	Rooiport females 2017	Rooiport females 2018
Dimethyl sulfone	8.38 ± 4.59	6.59 ± 3.69	6.27 ± 4.93	6.92 ± 2.48	10.18 ± 4.87	5.70 ± 2.67	11.63 ± 4.38	5.7 ± 2.67	11.6 ± 4.38
Formic acid	2.34 ± 1.43	1.86 ± 0.73	1.89 ± 0.58	1.83 ± 0.93	2.82 ± 1.81	1.36 ± 0.26	2.82 ± 0.66	1.4 ± 0.26	2.8 ± 0.66
Fumaric acid	0.80 ± 0.22	0.73 ± 0.22	0.82 ± 0.26	0.63 ± 0.12	0.87 ± 0.21	0.74 ± 0.19	0.94 ± 0.18	0.7 ± 0.19	0.9 ± 0.18
Glutamic acid	22.78 ± 4.77	22.48 ± 6.73	24.87 ± 5.23	20.09 ± 7.77	23.07 ± 1.57	19.66 ± 5.83	21.95 ± 4.44	19.7 ± 5.83	22.0 ± 4.44
Glutamine	34.59 ± 10.38	29.90 ± 10.63	34.29 ± 10.38	25.52 ± 9.91	39.29 ± 8.09	24.82 ± 8.28	29.77 ± 4.45	24.8 ± 8.28	29.8 ± 4.45
Hippuric acid	13.16 ± 4.57	14.11 ± 5.37	14.87 ± 7.83	13.34 ± 1.49	12.22 ± 3.65	13.55 ± 6.82	13.19 ± 1.85	13.6 ± 6.82	13.2 ± 1.85
Histidine	9.22 ± 2.13	8.37 ± 2.28	9.26 ± 2.50	7.48 ± 1.86	10.08 ± 1.67	7.22 ± 1.79	8.89 ± 1.02	7.2 ± 1.79	8.9 ± 1.02
Isoleucine	12.86 ± 3.59	12.89 ± 4.91	14.95 ± 4.43	10.82 ± 4.89	12.83 ± 1.77	10.08 ± 3.39	14.67 ± 4.02	10.1 ± 3.39	14.7 ± 4.02
Lactic acid	1381.39 ± 543.26	1292.33 ± 637.49	1512.94 ± 682.80	1071.71 ± 571.40	1470.45 ± 446.14	1390.67 ± 488.05	1877.59 ± 365.72	1390.7 ± 488.05	1877.6 ± 365.72
Leucine	14.54 ± 3.91	13.43 ± 4.94	15.36 ± 5.14	11.50 ± 4.38	15.65 ± 2.26	10.60 ± 3.44	17.48 ± 6.28	10.6 ± 3.44	17.5 ± 6.28
Lysine	11.13 ± 6.50	8.14 ± 7.91	12.47 ± 7.87	3.81 ± 5.64	14.12 ± 2.61	4.02 ± 6.29	12.77 ± 8.91	4.0 ± 6.29	12.8 ± 8.91
Mannose	6.78 ± 1.49	6.43 ± 1.95	6.81 ± 1.76	6.05 ± 2.26	7.14 ± 0.77	5.43 ± 1.60	7.19 ± 1.38	5.4 ± 1.60	7.2 ± 1.38
myo-Inositol	11.38 ± 3.53	10.76 ± 3.79	11.30 ± 4.65	10.21 ± 3.16	12.00 ± 3.32	8.23 ± 2.40	14.43 ± 1.82	8.2 ± 2.40	14.4 ± 1.82
N,N-Dimethylglycine	0.48 ± 0.28	0.55 ± 0.36	0.44 ± 0.19	0.66 ± 0.48	0.42 ± 0.16	0.54 ± 0.46	0.61 ± 0.24	0.5 ± 0.46	0.6 ± 0.24

Metabolite	All giraffes	Females	Non-pregnant females	Pregnant females	Males	Rooiport 2017	Rooiport 2018	Rooiport females 2017	Rooiport females 2018
Phenylalanine	9.14 ± 1.84	8.36 ± 2.16	9.30 ± 2.38	7.42 ± 1.61	9.91 ± 1.08	7.28 ± 1.81	9.08 ± 1.19	7.3 ± 1.81	9.1 ± 1.19
Pyruvic acid	15.26 ± 6.32	12.26 ± 5.84	14.67 ± 7.33	9.85 ± 2.92	18.26 ± 5.49	10.15 ± 2.97	20.83 ± 8.90	10.1 ± 2.97	20.8 ± 8.90
Succinic acid	8.11 ± 2.51	8.13 ± 3.31	9.12 ± 3.21	7.14 ± 3.44	8.08 ± 1.55	7.03 ± 3.65	9.38 ± 1.18	7.0 ± 3.65	9.4 ± 1.18
Threonine	18.13 ± 7.50	14.26 ± 7.60	18.17 ± 8.56	10.34 ± 4.30	22.00 ± 5.26	11.96 ± 8.44	20.03 ± 6.65	12.0 ± 8.44	20.0 ± 6.65
Tyrosine	6.59 ± 1.60	5.95 ± 1.86	6.65 ± 2.01	5.25 ± 1.57	7.22 ± 1.03	4.84 ± 1.25	7.70 ± 2.14	4.8 ± 1.25	7.7 ± 2.14
Valine	29.28 ± 8.43	27.92 ± 10.96	31.80 ± 11.87	24.05 ± 9.58	30.63 ± 5.07	21.00 ± 5.03	33.13 ± 9.76	21.0 ± 5.03	33.1 ± 9.76

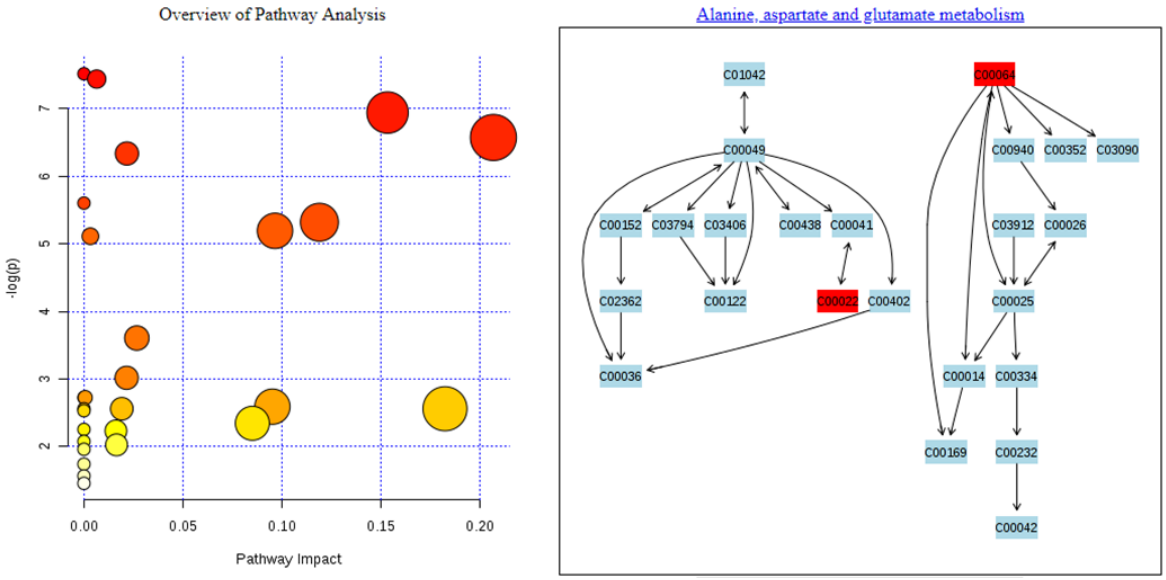


Figure 2.2: MetaboAnalyst pathway analysis plot based on the significant metabolites for sex showing the pathway for the largest red dot located at the pathway impact value of 0.20.

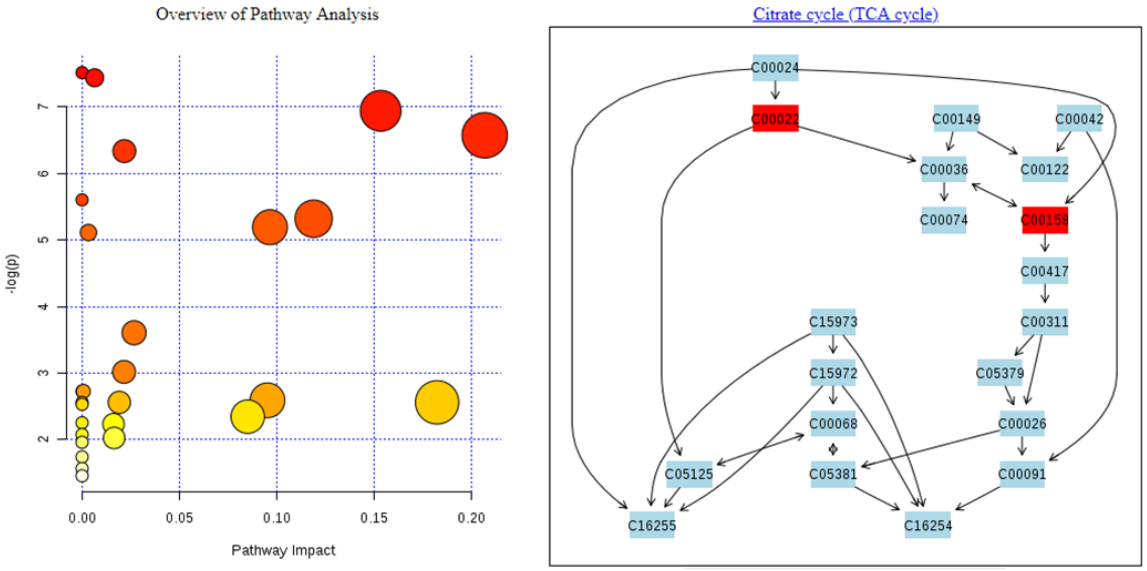


Figure 2.3: MetaboAnalyst pathway analysis plot based on the significant metabolites for sex showing the pathway for the second largest red dot located at the pathway impact value of 0.15.

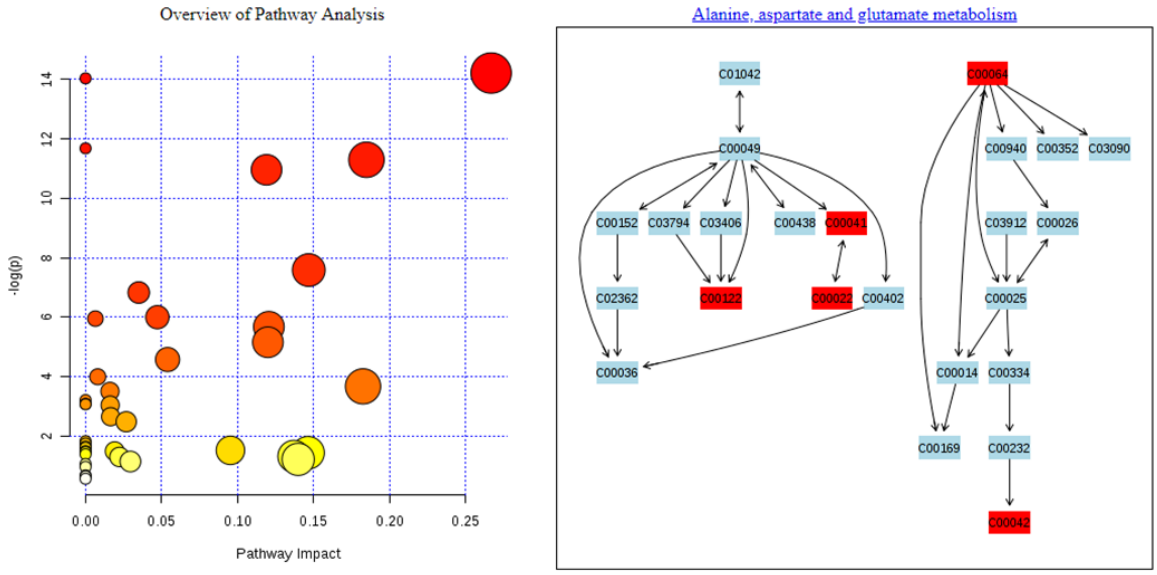


Figure 2.4: MetaboAnalyst pathway analysis plot based on the significant metabolites for year (Rooiport male and females) showing the pathway for the largest red dot located at the pathway impact value of above 0.25.

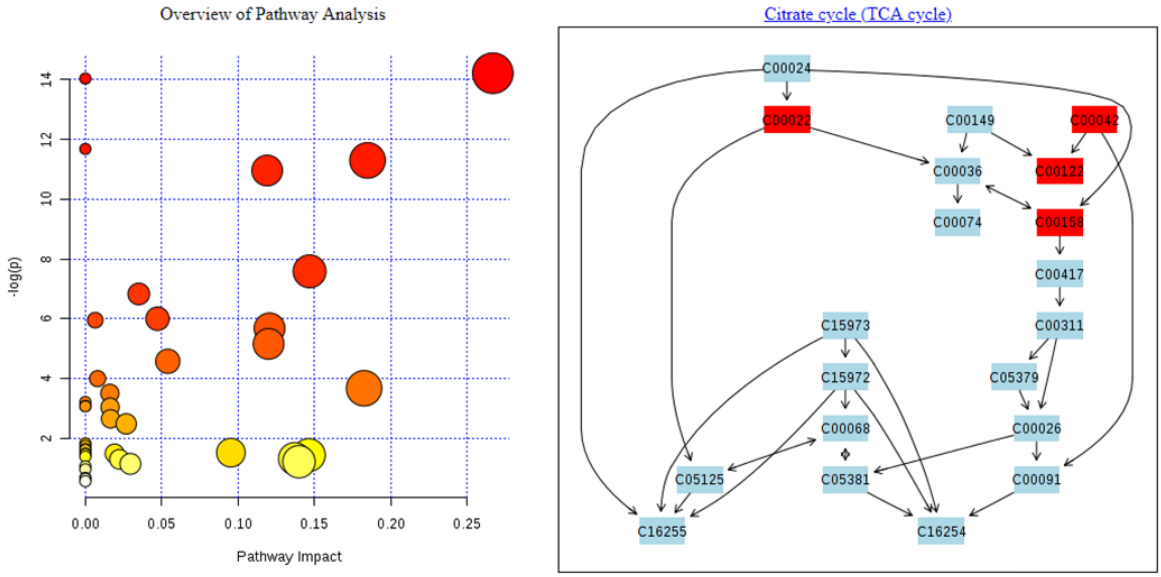


Figure 2.5: MetaboAnalyst pathway analysis plot based on the significant metabolites for year (Rooiport male and females) showing the pathway for the second largest red dot located at the pathway impact value of 0.18.

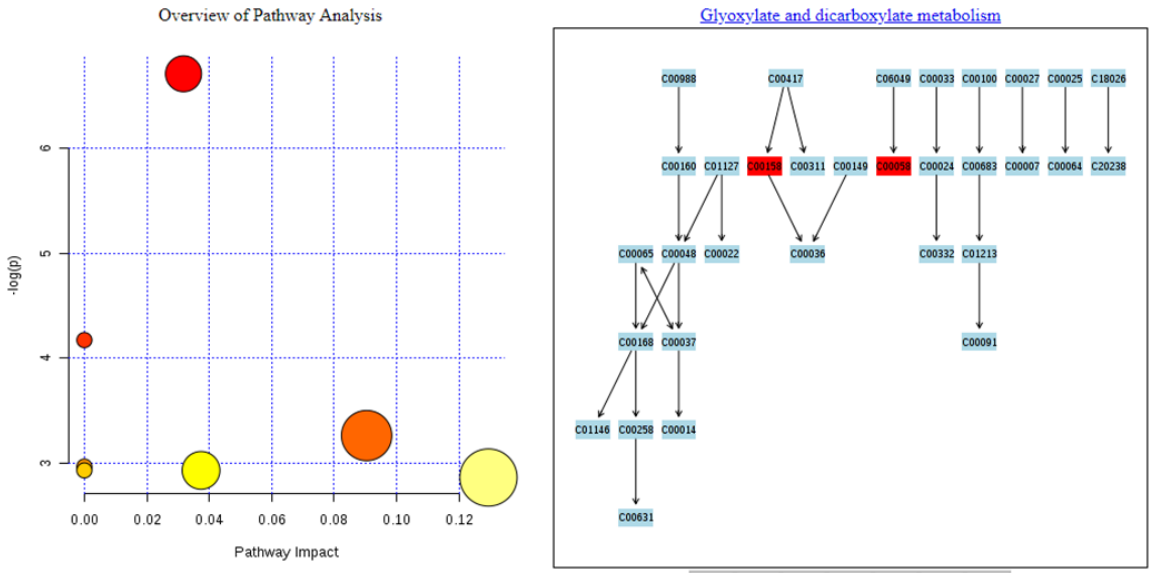


Figure 2.6: MetaboAnalyst pathway analysis plot based on the significant metabolites for year (Rooiport females) showing the pathway for the red dot located at the pathway impact value of 0.03.

2.4. Discussion

Statistical analysis showed some significant differences between male and female giraffes and year of sampling. These differences will be discussed below.

2.4.1. Serum metabolome differences based on sex.

There are significant differences in serum metabolites between the male and female giraffes, specifically pyruvic acid, citric acid, creatine, threonine, phenylalanine and glutamine. The concentrations of these metabolites were higher in male giraffes as seen in table 2.1. When compared to the pregnant and non-pregnant lactating female giraffes, the male serum metabolite concentrations seem to be more similar to the non-pregnant lactating female giraffes.

Sex differences of serum metabolites in mammals was not available in literature. The only report available for inter species comparison was a study on plasma metabolites in humans which showed gender differences (Trabado et al., 2017). In that study, gender differences were reported between male and female human plasma, however, the individual male and female plasma metabolite values were not listed. The differences were described in the

discussion, as human male plasma having had a higher concentration of a substantial number of metabolites when compared to female human plasma. Amino acids in general showed higher concentrations in human males when compared to female human plasma. Glycolysis and pyruvate metabolism showed higher activity in human males when compared to human females. The higher glucose concentrations in male human plasma was said to be due to the general physiological differences between genders. The higher concentrations of many of the metabolites, as well as the higher activity of the glycolysis and pyruvate metabolism, may also be due to physiological differences between the sexes (Trabado et al., 2017).

Amino acids and citric acid cycle related metabolites were detected at higher concentrations in male giraffes when compared to female giraffes. The alanine, aspartate, and glutamate metabolism and citric acid cycle were more active for male giraffes (Figure 2.2 and Figure 2.3) because the metabolic demands of the female giraffes were greater due to lactation and pregnancy. A higher activity of these pathways was expected for the male giraffes.

2.4.2. Serum metabolome differences based on year - nutrition

Differences in serum metabolic concentrations were observed for year of sampling. The most likely explanation why year of collection might have affected the serum metabolomes of the giraffes, was nutrition, as derived by the weather data and condition of the veld. The nutritional composition of the potential food was not analysed, however, the state of such can be derived from the climatic conditions. South Africa has been plagued by drought for about seven years. Weather data obtained from weather stations around the study site at Rooipoort showed that the annual rainfall, as well as the rainfall from one to five months prior to sample collection, of the 2018 season was much lower, and the average temperature higher, than the 2017 season (see Appendix C). This more than likely had a detrimental effect on the plants, specifically the trees that the giraffes browsed, that bore almost no leaves at the date of sample collection. The alanine, aspartate, and glutamate metabolism, citric acid cycle, and the glyoxylate and dicarboxylate metabolism (Figure 2.4 to Figure 2.6) were shown to be important pathways discerning the differences between the 2017 giraffe group and 2018 giraffe group and nutrition is responsible for these differences.

Other researchers have shown that leaf nutrient content of savanna trees in South Africa depended on water availability, specifically on regular rainfall (Barbosa et al., 2014). After receiving rainfall, young leaves appear, which have a high protein content. In a study on the giraffe seasonal utilisation of leaves, *Senegalia caffra* was said to be the most important and most utilised food plant. Seasonal changes in protein content was noted and the new growth leaves had the highest protein content. The percentage utilised by the giraffes correlated with

the seasonal changes in protein percentage. *Ziziphus mucronata* was also an important preferred food plant and was mostly utilised in the wet warm season. Crude protein content and moisture content were positively correlated with percentage utilization in most of the plant species studied (Sauer et al., 1982).

Many of the parameters of significant difference with $p < 0.05$, are amino acids as shown in Table B5. The 2018 giraffe samples had higher concentrations of the amino acids alanine, glutamine, histidine, leucine, phenylalanine, threonine, and tyrosine when compared to the 2017 giraffe group. In dairy cows, an increase in dietary protein was shown to elevate serum amino acid levels (Abu-Ghazaleh et al., 2001; Korhonen et al., 2002).

The higher concentration of amino acids in the 2018 giraffe group suggests a higher availability of protein from the diet. However, the nutrient deprived browsing, described above, suggests that this may not be the case. What was more likely, is that the nutrient deprived browsing may have left the giraffes nutritionally stressed, with resulting higher cortisol concentrations. Cortisol promotes protein breakdown in muscle and this increases the circulating amino acids in blood (Thung & Norwitz, 2009). Muscle breakdown of the giraffes from the 2018 group, due to nutritional stress, may be the cause of the increased amino acid concentrations.

Another possible reason for the increased amino acid concentrations is the consumption of non-leaf plant materials. Giraffes favour the leaves of *Senegalia caffra* trees (Parker, 2005), but, due to the potentially limited amount of leaves available in 2018, the giraffes might have eaten different parts of the plants, such as twigs and stems. Nutritional assessment of *Senegalia caffra catechu willd* showed that the stems, pods, and bark all contained higher protein concentrations than the leaves (Verma et al., 2014). The higher protein concentrations of these stems and pods as food may have elevated the serum amino acid concentrations in the 2018 giraffe group.

2.4.3. Serum metabolome differences based on year – temperature

In 2.4.2 it was attempted to explain the differences in observed serum metabolite concentrations between years by nutrition. However, the weather conditions also involved increased temperatures, which may also cause stress, and lead to changes in metabolite concentrations. Temperature data from weather stations around Rooiport reserve as seen in Tables C3 and C4 showed that in 2018 the average temperature for the month of sampling was higher than in 2017. The average maximum temperature for the month of sampling in

2017 was 27.4°C, while in 2018 it was 33.8°C. The 33.8°C probably caused greater heat stress on some of the giraffes than the 27.4°C. Heat stress caused changes in serum metabolite concentrations in Ewes (*Ovis aries*) (Farman et al., 2018) and in dairy cattle (Kekana et al., 2018).

It is also important to note the effect of cold temperatures on captive giraffes. Chronic energy malnutrition, increased energy demands due to cold weather, poor nutrition, and intake problems have caused energy deficiencies in captive giraffes. Hypoglycaemia has been reported for giraffes which have succumbed in the wintertime (Potter & Clauss, 2005). During a higher energy demand, dairy cows enter a state of ketosis, using the body reserves for energy. The energy imbalance experienced in cows is discussed in section 1.3.7. The brain requires a continual supply of glucose from the blood (Mergenthaler et al., 2013) and sustained hypoglycaemia causes brain death due to fuel deprivation (Cryer, 2007). Ketone bodies were not found in the collapsed giraffes and it was suggested that giraffes are unable to use their muscle or body reserves for energy and rely on carbohydrates for their energy supply (Potter & Clauss, 2005). The giraffes in that study were located at Auckalnd zoo, New Zealand and were fed an alfalfa hay diet. It was proposed that the giraffes did not digest enough of this hay to meet their energy needs. It was assumed that the giraffes were energy deficient for a long period before winter time. The cold temperatures in winter worsened their condition, so that the increased energy demands, due to the cold weather, then caused the giraffes to collapse. Giraffes are well adapted to hot environments and their body shape causes heat loss due to the high body surface area (Mitchell & Skinner, 2010). Giraffes are heterothermic and lower temperatures will cause an increased energy demand in order to maintain a normal body temperature (Potter & Clauss, 2005).

2.4.4. Serum metabolome differences based on year – energy deficiency

Nurturing a foetus and a young growing calf as a simultaneous reproductive strategy can cause a depletion of body nutrient reserves in the giraffes (Deacon et al., 2015). In a study on the 4 biofluids' metabolomics relationships in dairy cows, the citric acid cycle was said to be up-regulated for the lactating group when compared to the non-lactating group and this was reported for the metabolites in the rumen fluid, serum, milk and urine (Sun et al., 2017). The citric acid cycle was reported to be an enriched pathway for all four biofluids. The lactating group and non-lactating group were fed the same diet with the same energy density. Lactogenesis and foetal needs increase the cow's energy requirements and the nutritional stress during lacatation caused upregulation of the citric acid cycle in dairy cows. The level of

citric acid was significantly higher in the lactating dairy cows and may enhance energy by participating in the citric acid cycle (Sun et al., 2017). This may also be the case for the giraffes under study. There may also have been nutritional stress due to less rainfall being received for 2018 when compared to 2017. The nutrional stress may have caused an upregulation of the citric acid cycle.

The transition period is also a time during which the metabolome is affected. In cows, the transition period is the time frame formed by the three weeks prior to calving until three weeks postpartum. Lactogenesis and foetal needs increase the mammal's energy requirements. A mammal's metabolism can compensate by making use of body reserves when there is an imbalance of energy for a short period of time. This imbalance can affect the metabolite balance in both blood and milk. (Bezerra, et al., 2014). The age of the giraffe calves, 2.5-4.5 months, fall beyond the transition period, so that this is unlikely to have affected the giraffe metabolite concentrations.

2.4.5. Serum metabolome comparison between species

There is very little information available on serum metabolites in mammals and comparisons cannot be made as entire serum metabolomes are not available in literature. The human serum metabolome was available, and a comparison was made with the giraffe serum metabolome in Table B14 in appendix B. The human metabolite data was obtained from healthy subjects and most of the metabolites in the study fall in range with data from the literature listed in that study. The subjects in the study included males and females and the metabolites were detected by NMR (Psychogios et al., 2011).

Human serum was very different from giraffe serum as seen in the higher concentrations of listed metabolites. Amino acid concentrations in human serum were much higher than giraffe serum. For example, the amino acid alanine was present in much higher concentrations in human serum (427.2 ± 84.4 µM) vs giraffe serum (124.11 ± 32.71 µM). The citric acid cycle related metabolite citric acid is also much higher in human serum (114.2 ± 27 µM). The other citric acid cycle metabolites, fumaric acid and succinic acid, are either absent or were present at levels below detection in human serum. Shortcomings in the methods arise for many reasons including separation difficulties, compound solubility, and compound stability (Psychogios et al., 2011).

When female giraffe serum is compard to that of healthy dairy cows (Marczuk et al., 2018), the amino acid concentrations are much lower. For example, giraffe serum lysine (8.14 ± 7.91 µM) is much lower than healthy dairy cow lysine (74.36 ± 7.79 µM). Giraffe serum alanine

(109.43 ± 34.28 µM) seems to be more in the range of that in healthy dairy cows (190.91 ± 45.28 µM).

2.5. Conclusion

In this chapter, NMR was used to analyse the serum metabolome of male, non-pregnant lactating female and pregnant lactating female giraffes. There were significant differences in the serum metabolome for sex and year of sampling. Pregnancy status did not show any significant differences. The significant differences between year 2017 and 2018 suggest that the giraffe serum metabolome was affected by a change in diet due to environmental conditions, such rainfall, or stress due to high temperatures. This affected the citric acid cycle as well as amino acid metabolism. A significant difference ($p<0.05$) was noted for amino acids, such as glutamine, and energy related metabolites, such as citric acid, between the 2017 giraffe group and 2018 giraffe group. A small number of giraffes were available (n=11) for this study, specifically regarding the comparison over years (n=2). It would be of interest to do the study with larger numbers. However, it is very rare that a large group of animals would be available simultaneously for such an experimental design. The following chapter aims to determine the milk metabolome of the female giraffes.

Chapter 3 - Metabolites in giraffe milk

3.1. Introduction

Milk metabolites may be associated with milk nutritional quality. In a study on the metabolite profile of Holstein dairy cows (Melzer et al., 2013), Pearson correlation coefficients showed certain milk metabolites were correlated with milk nutrients such as protein content. Amino acid levels may also be related to lactose levels, because, amino acids are precursors of the protein α-lactalbumin, which is necessary for the synthesis of lactose (Melzer et al., 2013; Brew et al., 1968).

The milk metabolome can also indicate disorders, for example, a high amino acid and sodium content together with a low lactose content, indicates that a cow has mastitis (Ogola et al., 2007; Pohn et al., 2009). The milk metabolome is likely to differ between species, and also between individual subjects, and may change as lactation progresses (Klein et al., 2010; Smilowitz et al., 2013; Melzer et al., 2013; Zhang et al., 2015).

Many metabolites in milk have their origin in the serum, either from the uptake of nutrients in the intestine, or the metabolism of the organism (Lämmerhofer & Weckwerth, 2013; Bagchi et al., 2015). The milk cells, on the other hand, have their own metabolism and purpose made nutrient synthesis system, so that the milk metabolome differs substantially from the blood metabolome (Frandson et al., 2009). The fermentation of carbohydrates by rumen microorganisms produce fermentation products such as acetone, ethanol, butanol, and lactic acid (Darwin et al., 2018), which are taken up into the blood, and later transferred to the milk synthesising cells.

The milk metabolome of ruminants, specifically cow and goat, have been reported (Klein et al. 2010; Melzer et al. 2013; Caboni et al. 2016). These two species are of the taxonomic order of the Bovidae. Metabolites in giraffe, order Giraffidae, milk have not yet been reported, which is the aim of this chapter. Since the giraffe is one of a few species that may fall pregnant while nursing a calf (Deacon et al., 2015), this presented an interesting condition, i.e. milk of non-pregnant and pregnant lactating giraffes.

3.2. Materials and methods

3.2.1. Study site and sample collection

The study site, subjects and ethical aspects were described in Chapter 2, section 2.2.1.Milk was collected from sedated female giraffes by palpation of the teats. Milk from individual teats was collected separately and immediately frozen at -20°C until analysed. Each teat was milked

out, in order to obtain a whole milk sample, and not a first milk sample with different nutrient content (Iverson & Oftedal, 1995). Milk was thawed in a water bath at 39°C, mixed by swirling and subdivided into smaller volumes according to the volumes required for each analysis.

3.2.2. Sample preparation for NMR analysis

The milk samples were filtered using Amicon Ultra – 2mL centrifugal units with 10kDa membrane filters (Merck; Ref UFC201024). The rest of the preparation was the same as described for the serum in section 2.2.2.

3.2.3. ^{1}H-NMR analysis

NMR spectroscopy was carried out as described for the serum in section 2.2.3.

3.2.4. Statistical analysis

The statistical analysis was carried out as described in section 2.2.4.

3.2.5. MetaboAnalyst pathway analysis

The pathway analysis was carried out as described in section 2.2.5.

3.3. Results

An example of ^{1}H-NMR spectra of giraffe milk is shown in Figure 3.1. The mean concentrations of the metabolites (µM) in the milk of giraffes are shown in Table 3.1. Figure 3.2 shows the results from the MetaboAnalyst pathway analysis. Statistical analysis results are shown in Table B4 in the appendix. There were no significant differences for pregnancy status. There were many milk metabolites which showed a significance of p=0.0528 for year of sampling. The PCA plots showed no separation between the groups. These plots are labelled Figure B5 and B6 in appendix B. F9 was excluded from the results, because the high amino acid and sodium content as well as a low lactose content in the milk indicated that the giraffe may have had mastitis (Ogola et al., 2007; Pohn et al., 2009). AMP (adenosine monophosphate), ADP (adenosine diphosphate), and ATP were excluded from the results due to multiple zero values for most of the giraffes.

Table B4 shows the p values based on year of sampling for milk metabolites. The metabolites that differed at p=0.528 (Citric acid, niacinamide, uridine, lactose, phosphocholine, succinic acid, 2-Ketoglutaric acid, N-Acetylgalactosamine, creatine, pyruvic acid, fumaric acid, UDP, orotic acid, creatinine, glycerophosphocholine, hippuric acid, betaine, UDP-Glucose, butyric acid, UDP-Galactose, UDP-N-Acetylgalactosamine, and UDP-N-Acetylglucosamine), were subjected to the MetaboAnalyst pathway analysis, and are shown in Figure 3.2.

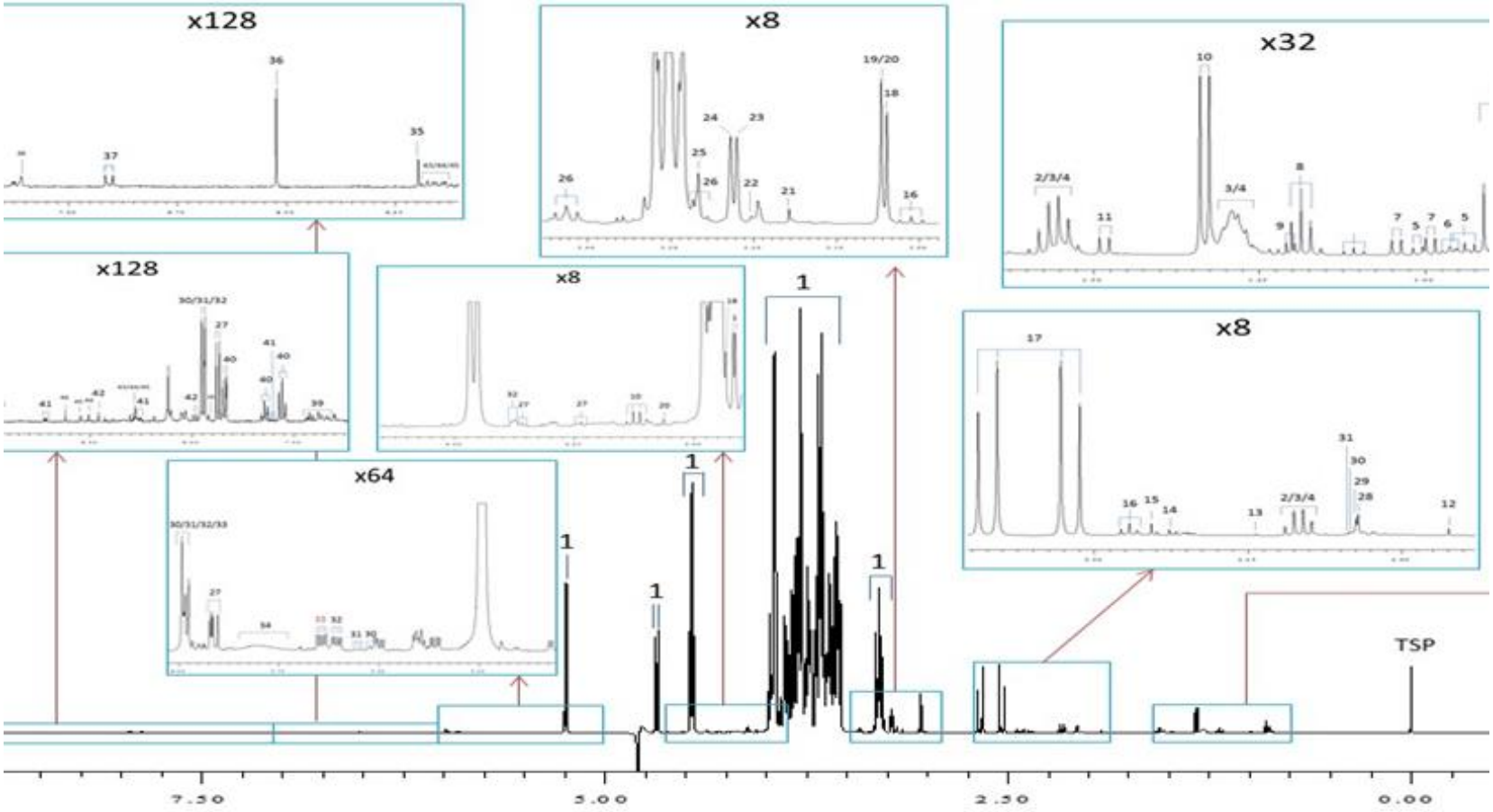


Figure 3.1 = [1]H NMR spectra for giraffe milk. Lactose (3,30 dd, 3,53-3,99, 4,46 d, 4,68 d, 5,24 d); 2 = Butyric acid (0.89 t, 1.56 h, 2.16 t); 3 = Caproic acid (0.87 t, 1.29 bs, 1.55 p, 2.17 t); 4 = Caprylic acid (0.86 t, 1.29 bs, 1.54 p, 2.17 t); 5 = Isoleucine (0.94 t, 1.01 d); 6 = Leucine (0.96 t); 7 = Valine (0.99 d, 1.04 d); 8 = Ethanol (1.18 t); 9 = 3-Hydroxybutyric acid (1.20 d); 10 = Lactic acid (1.33 d, 4.12 q); 11 = Alanine (1.48 d); 12 = Acetic acid (1.92 s); 13 = Acetone (2.23 s); 14 = Pyruvic acid (2.37 s); 15 = Succinic acid (2.40 s); 16 = 2- Ketoglutaric acid (2.44 t, 3.01 t); 17 = Citric acid (2.60 AB); 18 = Creatine (3.04 s, 3.93 s); 19 = Creatine phosphate (3.05 s); 20 = Creatinine (3.05 s, 4.06 s), 21 = Dimethyl sulfone (3.15 s); 22 = Choline (3.20 s); 23 = Phosphocholine (3.22 s); 24 = Glycerophosphocholine (3.23 s); 25 = Betaine (3.26 s); 26 = Taurine (3.27 t, 3.42 t); 27 = Uridine (4.23 t, 4.36 t, 5.91 d, 5.92 d, 7.88 d); 28 = N-Acetylglucosamine (2.07 s); 29 = N-Acetylgalactosamine (2.075 s); 30 = UDP-N-Acetylglucosamine (2.085 s, 5.52 dd, 5.98 dd, 7.95 d); 31 = UDP-N-Acetylgalactosamine (2.09 s, 5.55 dd, 5.98 dd, 7.95 d); 32 = UDP-Glucose (4.37 m, 5.60 dd, 5.98 dd, 7.95 d); 33 = UDP-Galactose (5.64 dd, 5.98 dd); 34 = Urea (5.80 bs); 35 = Orotic acid (6.20 s); 36 = Fumaric acid (6.52 s); 37 = Tyrosine (6.90 dd, 7.20 dd); 38 = Histamine (7.11 dd, 7.92 dd); 39 = Phenylalanine (7.38 m); 40 = Hippuric acid (7.56 t, 7.64 t, 7.84 d); 41 = Nicotinamide (7.61 dd, 8.26 dt, 8.72 dd, 8.94 dd); 42 = UDP (7.98 d); 43 = AMP (6.15 dd, 8.27 s, 8.62 s); 44 = ADP (6.15 dd, 8.27 s, 8.51 s); 45 = ATP (6.15 dd, 8.27 s, 8.55 s); 46 = Formic acid (8.46 s). Multiplicity: s − singlet; d − doublet; t – triplet; dd − double doublet; dt – double triplet; tt = triple triplet; q – quartet; p – pentet; h – hextet; m – multiplet; AB – AB system; BS – broad singlet. ND = not determined

Table 3.1: Metabolites (µM) in the milk of giraffes

	All giraffes	Non-pregnant	Pregnant	Rooiport 2017	Rooiport 2018
2-Ketoglutaric acid	607.59 ± 242.84	679.67 ± 233.60	391.36 ± 123.53	738.89 ± 169.24	282.73 ± 30.09
3-Hydroxybutyric acid	38.82 ± 69.35	47.13 ± 80.00	13.88 ± 2.77	12.12 ± 3.13	112.94 ± 137.32
Acetic acid	53.05 ± 58.14	63.37 ± 64.67	22.09 ± 13.75	38.70 ± 11.01	102.90 ± 128.04
Acetone	11.08 ± 2.83	11.46 ± 3.24	9.92 ± 0.29	9.65 ± 1.07	13.17 ± 4.89
Alanine	73.42 ± 40.98	87.31 ± 37.43	31.73 ± 10.96	84.45 ± 34.27	69.60 ± 64.52
Betaine	217.72 ± 112.82	239.03 ± 124.95	153.80 ± 11.90	272.37 ± 110.14	125.97 ± 27.45
Butyric acid	525.64 ± 554.83	619.24 ± 609.92	244.84 ± 290.81	782.75 ± 562.79	96.30 ± 80.75
Caproic/Caprylic acid	704.73 ± 716.11	823.95 ± 788.87	347.09 ± 370.23	1026.11 ± 740.65	179.41 ± 133.11
Citric acid	7607.51 ± 2572.69	8128.49 ± 2581.29	6044.58 ± 2549.88	8548.06 ± 1103.98	3829.31 ± 582.99
Creatine	971.55 ± 372.85	1058.88 ± 390.53	709.56 ± 165.89	1105.15 ± 260.53	478.74 ± 160.55
Creatinine	1439.73 ± 604.78	1596.38 ± 626.95	969.79 ± 77.78	1671.84 ± 572.92	768.26 ± 207.23
Formic acid	10.72 ± 4.01	11.93 ± 3.45	7.12 ± 4.28	11.86 ± 2.03	10.02 ± 8.39
Fumaric acid	37.61 ± 19.71	41.87 ± 20.21	24.83 ± 15.55	43.89 ± 14.61	12.22 ± 2.28
Glycerophosphocholine	418.03 ± 209.46	458.95 ± 230.73	295.29 ± 27.30	517.16 ± 198.35	211.29 ± 91.49
Hippuric acid	141.53 ± 84.47	163.21 ± 87.38	76.48 ± 21.97	180.20 ± 84.86	62.98 ± 2.88
Isoleucine	19.48 ± 14.45	22.31 ± 15.78	11.00 ± 4.93	16.59 ± 3.13	30.64 ± 32.70
Lactic acid	898.52 ± 945.87	1095.94 ± 1028.24	306.25 ± 202.32	761.21 ± 355.69	1603.16 ± 2036.42
Lactose	96894.17 ± 22331.92	97154.88 ± 25419.44	96112.05 ± 16082.05	109542.89 ± 5290.19	66179.36 ± 26249.17
Leucine	41.51 ± 29.08	45.68 ± 29.72	28.98 ± 32.89	44.96 ± 11.55	50.25 ± 62.97
N-Acetylgalactosamine	207.89 ± 46.43	207.87 ± 52.35	207.95 ± 37.19	221.27 ± 17.40	146.75 ± 49.36

	All giraffes	Non-pregnant	Pregnant	Rooiport 2017	Rooiport 2018
N-Acetylglucosamine	261.57 ± 163.59	262.57 ± 188.40	258.57 ± 99.16	338.94 ± 157.39	124.93 ± 89.83
Niacinamide	11.28 ± 4.15	12.66 ± 3.34	7.13 ± 4.35	13.44 ± 2.13	5.17 ± 1.58
Orotic acid	31.64 ± 13.88	34.50 ± 14.98	23.07 ± 5.49	37.25 ± 11.86	14.88 ± 6.08
Phenylalanine	37.56 ± 15.44	41.01 ± 15.98	27.22 ± 10.33	33.89 ± 2.96	46.45 ± 37.53
Phosphocholine	439.55 ± 122.71	443.17 ± 133.00	428.70 ± 129.00	500.40 ± 58.61	265.26 ± 102.13
Pyruvic acid	54.35 ± 18.43	59.06 ± 15.58	40.21 ± 25.10	63.80 ± 13.41	31.47 ± 12.73
Succinic acid	90.04 ± 34.02	101.71 ± 28.90	55.02 ± 25.55	106.89 ± 21.68	42.03 ± 7.18
Taurine	1209.99 ± 623.15	1324.83 ± 692.90	865.49 ± 34.36	1427.19 ± 717.04	797.18 ± 130.96
Tyrosine	5.32 ± 11.10	6.94 ± 12.64	0.46 ± 0.64	2.03 ± 2.09	16.21 ± 22.92
UDP	38.03 ± 13.15	42.40 ± 11.91	24.93 ± 6.58	41.69 ± 9.83	21.59 ± 1.86
UDP-Galactose	391.08 ± 441.17	474.08 ± 489.16	142.09 ± 26.66	528.41 ± 517.98	85.87 ± 52.84
UDP-Glucose	341.94 ± 265.93	399.99 ± 287.23	167.79 ± 40.50	450.51 ± 280.09	101.52 ± 53.22
UDP-N-Acetylgalactosamine	35.95 ± 50.51	43.61 ± 57.28	12.95 ± 6.48	50.53 ± 61.24	11.15 ± 3.94
UDP-N-Acetylglucosamine	76.41 ± 82.00	89.19 ± 92.86	38.06 ± 5.69	92.83 ± 103.23	37.27 ± 4.57
Uridine	240.45 ± 120.97	271.09 ± 120.24	148.56 ± 87.32	314.94 ± 74.09	83.46 ± 4.74
Valine	31.23 ± 23.60	35.84 ± 25.98	17.42 ± 3.96	25.52 ± 4.60	51.38 ± 51.99

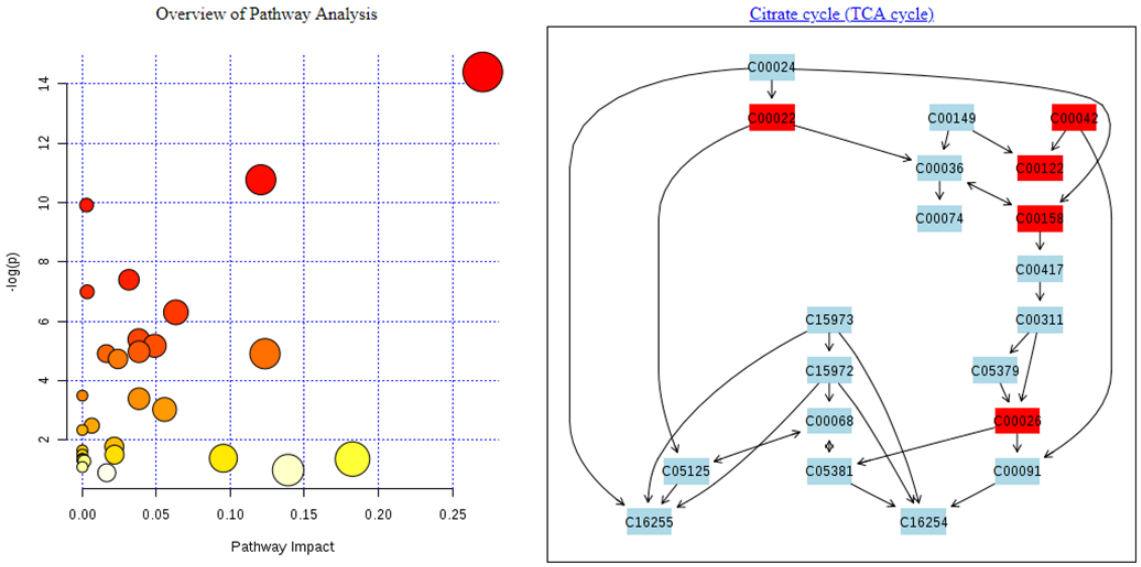


Figure 3.2: MetaboAnalyst pathway analysis plot for the most significantly different milk metabolites (p=0.0528) for year of sampling showing the pathway for the largest red dot located at the pathway impact value of above 0.27.

3.4. Discussion

There are no parameters that differed significantly at $p<0.05$ for the pregnancy status and year. However, there are several parameters that differ at $p=0.0528$, which deserve discussion regarding the differences observed for years.

3.4.1. Milk metabolome differences based on year

Although there are no significant differences ($p < 0.05$) between the metabolites of groups of giraffes, there are a number which significantly differ at $p=0.0528$. Specifically to be mentioned is the drop in lactose concentration for the 2018 group (66179.36 ± 26249.17 µM), compared to 2017 (109542.89 ± 5290.19 µM), as well as UDP-glucose and UDP-galactose which are involved with lactose synthesis (Anderson et al., 2014). It will be useful to discuss the effect of diet and rainfall on the concentration of this nutrient.

Based on the state of the nutrition between the years described in 2.4.3, giraffes may have had access to plant material with a lower fibre content and higher energy density in 2017. They may have had access to more digestible carbohydrates (Elsden, 1945; Orskov, 1986)

which favoured the production of propionic acid which supplied them with sufficient glucose to produce a higher amount of lactose.

In a study on five lactating Friesian diary cows and the production of volatile fatty acids in the rumen, production of rumen propionic acid more than doubled on a low roughage diet when compared to a conventional diet. Acetic acid and butyric acid production in the rumen decreased on the lower roughage diet (Sutton et al., 2003). The propionic acid produced in the rumen is then converted into glucose through gluconeogenesis in the liver (Frandson et al., 2009), and then released into the blood. Some propionate is metabolised through the rumen wall into lactic acid. The lactic acid is then also converted into glucose to be stored as glycogen, or to be released into the blood stream (Solaiman, 2010). In the milk cells, lactose is then synthesized from blood glucose, via UDP-glucose and UDP galactose (Frandson et al., 2009), which also explains the high content of the latter two in the milk (Table 3.1) (Anderson et al., 2014). Because propionate is completely used up, it does not appear in the milk.

The higher supply of propionate may also suggest that the citric acid cycle in the 2017 giraffe group was more active than in the 2018 giraffe group. The increase in glucose, because of the suggested increase in propionate, would have also meant that more glucose would be available to be converted to pyruvate and then into acetyl CoA to enter the citric acid cycle (Campbell & Farrell, 2012) and an increase in citric acid cycle related metabolites would be the result. The citric acid cycle related metabolites, 2-ketoglutaric acid, citric acid, fumaric acid, and succinic acid, are all higher in the milk of the 2017 (significance of $p=0.0528$) giraffe group when compared to the 2018 giraffe group and this supports the superior nutritional availability for the 2017 group. The MetaboAnalyst plots shown in Figure 3.2 indicate that the citric acid cycle was a preferable pathway which may distinguish female lactating giraffes based on year.

The differences of creatine and creatinine ($p=0.0528$) for year may indicate a higher energy demand. In a study on the 4 biofluids' metabolomics relationships in dairy cows, it was suggested that more creatine is needed to ease a serious negative energy balance in lactating dairy cows (Sun et al., 2017) and this could explain the lower amount of creatine in the milk of pregnant lactating (709.56 ± 165.89 µM) giraffes when compared to the non-pregnant lactating giraffes (1058.88 ± 390.53 µM). Creatine is an important intermediate metabolite in energy producing reactions. Muscles store energy as high-energy phosphocreatine. When there is a higher energy demand in the body the cells use ATP obtained from the reaction of ADP with phosphocreatine. Creatinine is a waste product of creatine and its levels are correlated with creatine levels (Johnston et al., 2016). Decreased amount of creatinine levels may also be

reflective of the decreased amount of creatine in pregnant lactating giraffes when compared to non-pregnant lactating giraffes.

As mentioned in section 2.4.3., the transition period causes an increased energy demand in cattle and this can cause an imbalance of energy for a short period of time. This imbalance can affect the metabolite balance in both blood and milk. (Bezerra, et al., 2014). The age of the giraffe calves, 2.5-4.5 months, fall beyond the transition period, so that this is unlikely to have affected the giraffe metabolite concentrations.

Hippuric acid and niacinamide also differ at p=0.0528 for year and they are rumen microbe related compounds appearing in giraffe milk. Changes in the concentrations of the rumen microbe related compounds niacinamide and hippuric acid in giraffe milk may just indicate changes in the rumen microbe populations due to the dietary changes affected by the browsing. Hippuric acid is a glycine conjugate of benzoic acid associated with intestinal microbiota degradation of specific dietary components (Lees et al. 2013), while niacinamide is a vitamin synthesized in the rumen by rumen microbes (Hungate, 1966).

3.4.2. Individual variation

There is variation in milk metabolite concentrations between individual giraffes. The large individual variation in metabolite concentration for each giraffe may be normal. Differences in metabolite concentrations in milk for individual subjects have been shown in human, panda, and cow's milk (Smilowitz et al., 2013; Zhang et al., 2015; Melzer et al., 2013; Klein et al., 2010). A high inter-individual variation of milk saccharides was also reported in the human milk metabolome (Smilowitz et al., 2013).

Variation in milk metabolite concentrations were reported for other animals. The metabolite composition in giant panda milk was shown to change over time and unique profiles were shown for the individual pandas (Zhang et al., 2015). In a study on the milk metabolomics of dairy cows, there were large differences in concentrations of energy related metabolites such as 3HBA and acetone. This indicates that individual animals cope differently with the metabolic stress during the lactation period (Klein et al., 2010).

3.4.3. Inter-species comparison of milk metabolites

Metabolites present in the milk of different species are shown in Tables A1 to A6 under appendix A. The giraffe milk metabolome is not particualry similar to any of the species listed in these tables. However, some of the metabolites such as histidine, cysteine, and

pantothenate are either absent or present at undetectable levels in the ruminant species as well as the giraffe. The most commonly occurring metabolites in the milk of the species listed in these tables, including giraffe, are valine, lactose, lactate, succinate, alanine, aspartate, phenylalanine, and isoleucine. Different analytical methods used to analyse the metabolites may likely be responsible for the differences. In the study on Holstein cows, the GC-MS analyses measured only part of the milk metabolome, and predominantly short-chain water-soluble metabolites were detected (Melzer et al., 2013). Therefore, only some metabolites may be absent for Holstein cows when compared to the study done on the Brown Swiss and Simmental cows, which used both NMR and GC-MS analyses (Klein et al., 2010).

A comparison of giraffe and cow milk metabolites is shown in Table B7 in appendix B. The metabolic pathways are also listed. The concentration of many of the amino acids present in giraffe milk are higher than those present in cow milk. The higher fibre content of hay in the partial mixed rations fed to the cows (Klein et al., 2010) could possibly attribute to the higher acetic acid content in cow milk (Perry, 1980; J. Moran, 2005) when compared to giraffe milk.

Some of the metabolites with p-values of 0.0528 include citric acid cycle related metabolites as seen in Table B4. Citric acid concentration is high in milk of both giraffes and cows. The citric acid cycle has been reported to be upregulated in cow milk production. Citric acid was also said to be significantly high and this enhances energy to ease the serious negative energy balance seen in lactating dairy cows (Sun et al., 2017). Creatinine is much higher in giraffe milk when compared to cow milk, while creatine is absent in cow milk. Some of the other metabolites from Brown Swiss cow and Simmental cow milk are also absent when compared to the giraffe milk metabolome. Metabolite concentrations of this cow milk were obtained from either GC-MS or NMR spectrawhich makes comparison difficult, as the sensitivity of the analytical techniques differ (Emwas, 2015).

3.4.4. Mammary gland metabolism

There are differences in the giraffe serum and milk metabolomes. Metabolites such as alpha-glucose and beta-glucose are found in serum but not in milk. Metabolites such as butyric acid and caproic acid are found in milk but not in serum. Many of the metabolites in serum are at lower concentrations than those in milk. For example, serum citric acid (28.39 ± 11.98) is at a much lower concentration than milk citric acid (7607.51 ± 2572.69). Serum metabolites such as amino acids are continuously regulated due to homeostasis. Interorgan amino acid transport is an important component involved in maintaining amino acid homeostasis (Brosnan, 2003) and because of this regulation, the correlations between serum and milk

nutrients may not be dependable. There may also be differences in the metabolites present for both milk and serum between species. For example, a study on the ketone bodies in the blood and milk of dairy cows reported that cow milk acetoacetate and 3HBA were present at lower concentrations when compared to cow serum (Enjalbert et al. 2001); however, the 3HBA in giraffe milk showed individual variation and is slightly higher in concentration when compared to giraffe serum and acetoacetate was not detected in giraffe milk and blood.

The metabolites which are present in milk and not serum show that milk cells have a metabolism of their own. The most obvious example is the production of lactose. The lactose metabolites UDP-glucose and UDP-galactose are present in giraffe milk and not giraffe blood (Anderson et al., 2014). Lactose is synthesized inside of the secretory cells of the mammary gland (Frandson et al., 2009). Another example is glycerophospholipid synthesis. Glycerophospholipids are located on the milk fat globule membrane (Contarini & Povolo, 2013) and are synthesized by the mammary epithelial cells (Wong, 1988). Phosphocholine and glycerophosphocholine are also present in giraffe milk and not giraffe blood. These two metabolites are involved in glycerophospholipid metabolism (Matsuda et al., 2017). Mammary cell glycerophospholipid synthesis pathways are common to other mammalian cells (Wong, 1988), however, these glycerophosolipid metabolites may not be exclusively derived from the blood due to their absence in giraffe serum and presence in giraffe milk. In a study on the major choline metabolites in rat milk, rat mammary epithelial cells, in primary culture, synthesized and secreted phosphocholine and glycerophosphocholine (Rohlfs et al., 1993). Other giraffe milk metabolites such as glutamic acid, glutamine, lysine, and threonine are absent in serum and this may be because their concentrations were below the detection limit (Emwas, 2015).

3.5. Conclusion

In this chapter, NMR was used to analyse the milk metabolome of non-pregnant lactating female and pregnant lactating female giraffes. There were no significant differences for pregnancy status and year of sampling. However, there were differences of metabolites at $p=0.0528$ specifically relating to the synthesis of lactose. The lower lactose concentration for the 2018 group (66179.36 ± 26249.17 µM), compared to 2017 (109542.89 ± 5290.19 µM), is due to dietary factors. The milk metabolites UDP-glucose and UDP-galactose differ at $p=0.0528$ and are also involved in lactose synthesis. Differences in giraffe milk creatine and creatinine may indicate a change in energy demand of the giraffes between years of sampling. A small number of giraffes were available for this study, specifically regarding the comparison

over years. It would be of interest to do the study with larger numbers. However, it is very rare that a large group of animals would be available simultaneously for such an experimental design.

Chapter 4 - Nutrient composition of giraffe milk and its relationship to serum and milk metabolomes

4.1. Introduction

The nutrient composition of giraffe milk was reported previously (Osthoff et al., 2017). However, the study involved only four giraffes. The lactation time ranged between 3 and 5 months, and pregnancy status of the animals was unknown. These giraffes were located at two different reserves of different vegetation types, but no statistical differences were noted between the milks of origin.

The current study provided an opportunity where milk from a group of giraffes could be obtained that forage on the same vegetation, of which lactation stage was precisely known, of which the state of a second pregnancy was known, and a sampling in a following year was possible. This is important because it is known that different forage (Chilliard et al., 2001) and stage of lactation (Nantapo et al., 2014) may affect milk composition in cows, specifically the fat composition. Furthermore, pregnancy creates a higher energy demand and this can affect the nutrient content in milk (Kara et al., 2013). This chapter aims to determine the nutrient content in the milk of non-pregnant lactating giraffes and pregnant lactating giraffes over two years.

4.2. Materials and methods

4.2.1. Study site and sample collection

The study site, subjects and ethical aspects were described in Chapter 2, section 2.2.1. Collection, storage and treatment of milk were described in Chapter 3, section 3.2.1.

4.2.2. Protein fractionation

Non-protein nitrogen (NPN) and whey proteins were fractionated by selective precipitation according to the method of Csapó et al (1996). For calculation of protein and whey content from nitrogen content, a conversion factor of 6.35 was used.

4.2.2.1. Total Nitrogen (TN) Determination

100 µL giraffe milk was placed into an Eppendorf tube. TN was determined by using LECO combustion analysis. The NPN percentage was subtracted from the TN percentage to obtain the total protein percentage.

4.2.2.2. Non-protein Nitrogen (NPN) Determination

200 µL 15% Trichloroacetic acid (TCA) was added to 100 µL Giraffe milk. The mixture was vortexed for 5 seconds and then centrifuged in a microcentrifuge centrifuge (Bio-Rad, South Africa) for 5 minutes at 7 000 rpm. The supernatant was collected and the NPN was determined by using LECO combustion analysis. The nitrogen percentage was obtained and multiplied by a dilution factor of 3 to calculate the nitrogen percentage in 100 µL giraffe milk.

4.2.2.3. Whey Protein Determination

100 µL H_2O was added to 100 µL giraffe milk. The mixture was vortexed for 5 seconds. 30 µL 10 % acetic acid (AcOH) was added. The mixture was vortexed for 5 seconds and allowed to stand for about 20 minutes. 30 µL 1 N AcOH was added. The mixture was vortexed and allowed to stand for 20 minutes. 250 µL H_2O was added to the mixture and the mixture was vortexed for 5 seconds. The mixture was allowed to stand for 1 hour. The mixture was then centrifuged. The supernatant was collected and the crude whey with NPN percentage was determined by using LECO combustion analysis. The true whey percentage was determined by subtracting the NPN percentage from the crude whey with NPN percentage. The casein percentage was then determined by subtracting the whey percentage from the total protein percentage.

4.2.3. Lipid extraction and fatty acid analysis

Total lipid from milk samples was quantitatively extracted, according to the method of Folch, Lees & Sloane-Stanley (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 moisture adsorbent. Total extractable fat content (EFC) was determined gravimetrically and expressed as % fat (w/w) per 100g 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 polytop (glass vial, with push-in top) under a blanket of nitrogen and frozen at –20°C until further analyzed.

Approximately 10 mg of total lipid (from 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 and 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). Column temperature was 40–230°C (hold 2 minutes; 4°C/minute; hold 10 minutes). Fatty acid methyl esters in hexane (1µl) 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-Aldrich (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 of all 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 mono-unsaturated 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.

4.2.4. Carbohydrate analysis

High performance Liquid Chromatography (HPLC) was used to determine the carbohydrate content of the milk samples. A Waters Breeze High Performance Liquid Chromatography system was used to determine carbohydrate content by means of Biorad Aminex 42C (3007.8 mm) and Waters Sugar Pak 1 (3007.8 mm) columns at 84°C with a differential refractive detector. Deionized water was used as the mobile phase. The mobile phase was eluted at 0.6 ml/min. Centrifugation was used to de-fat and de-proteinize the samples. Centrifugation took place at 3000 g in Ultrafree-CL (UFC4 LCC 25) filter devices (Millipore). Maltotriose, galactose, glucose, and lactose were used as standards. HPLC was used to monitor acid hydrolysis of the oligosaccharides. This was carried out at a final concentration of 1 N HCl at 40°C until the process was complete. Beta-galactosidase from *Aspergillus oryzae* (E.C. No. 3.2.1.23)

(Sigma), cellulase (h-glucan glycanohydrolase) from *Aspergillus niger* (E.C. No. 3.2.1.4) (Sigma) and heat stable a-amylase (E.C. No. 232-560-9) (Sigma) were the enzymes used. Over time, HPLC was used to monitor the hydrolysis products (Osthoff, et al., 2005).

4.2.5. Analysis of moisture, ash and minerals

A crucible was dried in a hot air oven and weighed. 0,5g of giraffe milk sample was accurately weighed in the crucible. The crucible was heated in an oven at 102°C for 60 minutes. The dry samples were weighed and moisture content determined. The sample was then placed in a muffle furnace at 550°C for about 2 hours. Nitric acid (2 parts water to one part nitric acid) was added to each sample to complete the digestion. The sample was then placed in the furnace until white ash was formed. The weight of ash was determined and ash content of milk calculated. The crucible was rinsed out with a nitric acid dilution and poured into a volumetric flask (50ml). This step was repeated 3 times. Lastly, distilled water was used to rinse the crucible and this water rinse was added to the volumetric flask. Distilled water was used to make up the 50 ml. The mineral content of the samples were analysed by ICP-OES (inductively coupled plasma mass spectrometry - optical emission spectroscopy) using a model Prodigy 7 (Teledyne Leeman Labs, IC) making use of the inductively coupled plasma method (plasma emission spectroscopy, 3120 B) (Eaton et al. 2005).

4.2.6. Statistical analysis

The statistical analysis was carried out as described in section 2.2.4. Pearson correlation coefficients were calculated using Excel 2016 (Microsoft, Redmond, Washington).

4.3. Results

The major nutrients in giraffe milk are shown in Table 4.1. The fatty acid composition of the milk fat in giraffe milk is shown in Table 4.2. The mineral composition of giraffe milk is shown in Table 4.3. In these three tables the average contents were calculated according to groupings, i.e. all giraffes, pregnancy status, origin and year. Data of these groups were statistically compared. The statistical analysis results are shown in Table B5 in the appendix. The PCA plots are labelled Figure B7 and B8. Figure B8 shows separation based on year. A correlation table (Table 4.4) shows a correlation of the giraffe milk and serum metabolites with the giraffe milk nutrients. Giraffe F9 was excluded from the results. The high amino acid (as

reported in chapter 3) and sodium content as well as a low lactose content in the milk indicated that the giraffe may have had mastitis (Ogola et al., 2007; Pohn et al., 2009).

Table 4.1: Major nutrients (g/100g) in giraffe milk according to pregnancy status, origin and year of collection

Nutrient	Giraffe average	Non-pregnant average	Pregnant average	Rooiport giraffes 2017 average	Rooiport giraffes 2018 average	Sandveld giraffe
Dry matter	13.64 ± 2.41	13.43 ± 2.25	14.17 ± 3.70	15.15 ± 1.61	10.76 ± 1.12	13.36 ± 0.003
NPN	0.15 ± 0.04	0.16 ± 0.03	0.14 ± 0.08	0.17 ± 0.03	0.10 ± 0.02	0.15 ± 0.07
Total Protein	3.75 ± 0.83	3.88 ± 0.92	3.37 ± 0.40	3.49 ± 0.88	3.84 ± 0.25	4.89 ± 0.35
Whey	0.82 ± 0.35	0.94 ± 0.29	0.47 ± 0.35	0.94 ± 0.22	0.70 ± 0.68	0.48 ± 0.07
Casein	3.15 ± 1.12	3.24 ± 1.27	2.90 ± 0.76	2.57 ± 0.85	3.64 ± 0.30	5.06 ± 0.57
Fat	6.61 ± 1.60	6.30 ± 1.02	7.69 ± 3.34	7.23 ± 1.95	5.35 ± 0.03	6.30 ± 0.11
Lactose	4.57 ± 0.79	4.64 ± 0.90	4.36 ± 0.40	5.07 ± 0.34	3.62 ± 0.64	3.94 ± 0.80
Galactose	0.04 ± 0.04	0.05 ± 0.03	0.00 ± 0.00	0.04 ± 0.04	-	-
Ash	1.08 ± 0.30	1.16 ± 0.33	0.88 ± 0.004	1.13 ± 0.40	1.03 ± 0.20	0.99 ± 0.263

Table 4.2: Fatty acid content (% of total) of giraffe milk according to pregnancy status, origin and year of collection

Common name:	Abbreviation	Giraffe average	Non-pregnant average	Pregnant average	Rooiport giraffes 2017 average	Rooiport giraffes 2018 average	Sandveld giraffe
Butyric	C4:0	0.48 ± 0.16	0.49 ± 0.18	0.45 ± 0.15	0.58 ± 0.14	0.30 ± 0.06	0.53 ± 0.03
Caproic	C6:0	1.37 ± 0.31	1.39 ± 0.35	1.31 ± 0.18	1.53 ± 0.22	1.07 ± 0.16	1.63 ± 0.04
Caprylic	C8:0	2.15 ± 0.40	2.18 ± 0.45	2.05 ± 0.02	2.32 ± 0.24	1.84 ± 0.29	2.58 ± 0.06
Capric	C10:0	6.42 ± 1.15	6.42 ± 1.31	6.40 ± 0.52	6.55 ± 0.68	5.93 ± 1.20	8.40 ± 0.31
Hendecanoic	C11:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lauric	C12:0	1.11 ± 0.10	1.12 ± 0.09	1.10 ± 0.17	1.11 ± 0.09	1.15 ± 0.10	1.21 ± 0.05
Tridecoic	C13:0	0.03 ± 0.04	0.03 ± 0.04	0.05 ± 0.04	0.01 ± 0.01	0.07 ± 0.01	0.10 ± 0.00
Myristic	C14:0	10.56 ± 1.27	10.38 ± 1.38	11.16 ± 0.74	10.37 ± 1.33	10.58 ± 1.56	12.17 ± 0.43

Common name:	Abbreviation	Giraffe average	Non-pregnant average	Pregnant average	Rooiport giraffes 2017 average	Rooiport giraffes 2018 average	Sandveld giraffe
Myristoleic	C14:1c9	0.12 ± 0.03	0.11 ± 0.02	0.16 ± 0.02	0.11 ± 0.03	0.14 ± 0.04	0.13 ± 0.00
Pentadecylic	C15:0	1.30 ± 0.24	1.25 ± 0.25	1.47 ± 0.09	1.17 ± 0.22	1.59 ± 0.06	1.41 ± 0.02
Pentadecenoic	C15:1c10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Palmitic	C16:0	25.26 ± 1.30	24.95 ± 1.32	26.32 ± 0.29	24.55 ± 1.25	26.18 ± 0.49	26.92 ± 0.11
Palmitoleic	C16:1c9	1.20 ± 0.25	1.17 ± 0.23	1.32 ± 0.42	1.14 ± 0.26	1.49 ± 0.18	1.14 ± 0.05
Phytanic	C20:0 3,7,11,15tetramethyl hexadecanoic acid	0.03 ± 0.02	0.02 ± 0.02	0.05 ± 0.00	0.03 ± 0.03	-	-
Margaric	C17:0	1.32 ± 0.72	1.42 ± 0.79	1.01 ± 0.35	0.91 ± 0.12	1.67 ± 0.57	1.14 ± 0.09
Heptadecenoic	C17:1c10	0.15 ± 0.08	0.14 ± 0.08	0.19 ± 0.08	0.10 ± 0.06	0.26 ± 0.01	0.17 ± 0.03
Stearic acid	C18:0	14.07 ± 1.32	13.95 ± 1.50	14.48 ± 0.18	13.65 ± 1.23	15.53 ± 1.29	14.30 ± 0.72
Elaidic	C18:1t9	0.13 ± 0.09	0.15 ± 0.10	0.08 ± 0.00	0.12 ± 0.02	0.08 ± 0.01	0.08 ± 0.00
Oleic	C18:1c9	27.07 ± 3.39	27.45 ± 3.37	25.74 ± 4.38	29.16 ± 1.62	23.82 ± 1.66	21.36 ± 0.66
Vaccenic	C18:1c7	0.36 ± 0.50	0.33 ± 0.53	0.44 ± 0.54	0.04 ± 0.01	0.85 ± 0.04	1.30 ± 0.06
Nonoadecanoic	C19:0	1.73 ± 1.04	2.01 ± 1.01	0.76 ± 0.30	1.84 ± 0.70	0.91 ± 0.51	0.82 ± 0.07
Linolelaidic	C18:2t9,12 (n-6)	0.97 ± 1.50	0.74 ± 1.28	1.79 ± 2.53	0.00 ± 0.00	3.31 ± 0.37	2.11 ± 0.06
Linoleic	C18:2c9,12 (n-6)	1.79 ± 1.19	1.92 ± 1.17	1.33 ± 1.64	2.59 ± 0.27	0.23 ± 0.06	0.20 ± 0.00
Arachidic	C20:0	0.27 ± 0.08	0.26 ± 0.09	0.28 ± 0.03	0.22 ± 0.05	0.37 ± 0.09	0.27 ± 0.01
γ-Linolenic	C18:3c6,9,12 (n-3)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Eicosenoic	C20:1c11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
α-Linolenic	C18:3c9,12,15 (n-3)	1.22 ± 0.40	1.16 ± 0.36	1.41 ± 0.64	0.97 ± 0.08	1.87 ± 0.01	1.40 ± 0.03
Conjugated linoleic acid	C18:2c9t11 (n-6) (CLA)	0.15 ± 0.05	0.15 ± 0.06	0.16 ± 0.04	0.12 ± 0.02	0.22 ± 0.04	0.22 ± 0.01
Heneicosanoic	C21:0	0.31 ± 0.35	0.36 ± 0.38	0.13 ± 0.12	0.46 ± 0.40	0.07 ± 0.02	0.04 ± 0.01
Eicosadienoic	C20:2c11,14 (n-6)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.00	0.01 ± 0.01
Behenic	C22:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Common name:	Abbreviation	Giraffe average	Non-pregnant average	Pregnant average	Rooiport giraffes 2017 average	Rooiport giraffes 2018 average	Sandveld giraffe
Eicosatrienoic	C20:3c8,11,14 (n-6)	0.09 ± 0.04	0.09 ± 0.05	0.09 ± 0.01	0.07 ± 0.02	0.13 ± 0.05	0.08 ± 0.01
Erucic	C22:1c13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
Eicosatrienoic	C20:3c11,14,17 (n-3)	0.00 ± 0.01	0.00 ± 0.00	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Arachidonic	C20:4c5,8,11,14 (n-6)	0.11 ± 0.04	0.11 ± 0.03	0.11 ± 0.10	0.08 ± 0.03	0.17 ± 0.01	0.10 ± 0.00
Tricosanoic	C23:0	0.03 ± 0.04	0.04 ± 0.04	0.03 ± 0.01	0.01 ± 0.02	0.08 ± 0.05	0.02 ± 0.01
Docosadienoic	C22:2c13,16 (n-6)	0.13 ± 0.13	0.16 ± 0.14	0.02 ± 0.03	0.18 ± 0.12	0.00 ± 0.00	0.00 ± 0.00
Eicosopentaenoic	C20:5c5,8,11,14,17 (n-3)	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.02 ± 0.01	0.02 ± 0.00
Lignoceric	C24:0	0.04 ± 0.04	0.04 ± 0.04	0.05 ± 0.01	0.02 ± 0.02	0.08 ± 0.04	0.04 ± 0.01
Nervonic	C24:1c15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Docosapentaenoic	C22:5c7,10,13,16,19 (n-3)	0.02 ± 0.02	0.01 ± 0.02	0.03 ± 0.04	0.00 ± 0.00	0.05 ± 0.02	0.05 ± 0.01
Docosahexanoic	C22:6c4,7,10,13,16,19 (n-3)	0.01 ± 0.01	0.00 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.03 ± 0.00
Fatty acid ratios:							
Saturated Fatty Acids	SFA	66.26 ± 2.49	66.07 ± 2.79	66.90 ± 1.36	65.33 ± 2.19	66.68 ± 1.68	70.96 ± 0.78
Mono Unsaturated Fatty Acids	MUFA	29.03 ± 2.87	29.34 ± 2.94	27.92 ± 3.33	30.66 ± 1.82	26.63 ± 1.50	24.19 ± 0.69
Poly Unsaturated Fatty Acids	PUFA	4.71 ± 1.19	4.58 ± 1.07	5.18 ± 1.96	4.01 ± 0.40	6.70 ± 0.18	4.85 ± 0.10
Omega-3 Fatty Acids	n-3	1.25 ± 0.43	1.19 ± 0.38	1.47 ± 0.68	0.97 ± 0.08	1.94 ± 0.02	1.50 ± 0.03
Omega- 6 Fatty Acids	n-6	3.46 ± 0.78	3.40 ± 0.72	3.70 ± 1.28	3.03 ± 0.34	4.76 ± 0.21	3.34 ± 0.10

Table 4.3: Minerals (mg/100g) in giraffe milk according to pregnancy status, origin and year of collection

Minerals	Giraffe average	Non-pregnant average	Pregnant average	Rooiport giraffes 2017 average	Rooiport giraffes 2018 average	Sandveld giraffe
Calcium (Ca)	222.38 ± 44.20	217.67 ± 51.27	236.54 ± 0.84	229.66 ± 51.29	203.19 ± 46.32	224.38 ± 11.62
Magnesium (Mg)	14.73 ± 2.89	14.55 ± 3.39	15.28 ± 0.06	16.22 ± 1.82	12.51 ± 3.86	11.72 ± 0.38
Sodium (Na)	82.89 ± 22.15	84.27 ± 23.95	78.73 ± 22.82	98.04 ± 8.44	61.38 ± 1.71	50.12 ± 5.13
Potassium (K)	87.64 ± 19.11	87.58 ± 22.50	87.81 ± 5.13	97.44 ± 12.88	75.52 ± 22.52	62.89 ± 0.28
Cadmium (Cd)	0.26 ± 0.07	0.28 ± 0.07	0.22	0.26 ± 0.07	<0.003	<0.003
Chromium (Cr)	1.60 ± 0.47	1.78 ± 0.28	0.87	1.60 ± 0.47	<0.020	<0.020
Copper (Cu)	0.45 ± 0.10	0.49 ± 0.10	0.35 ± 0.04	0.48 ± 0.12	0.38 ± 0.08	0.48 ± 0.16
Iron (Fe)	4.16 ± 3.17	4.73 ± 3.45	2.44 ± 1.62	6.21 ± 1.84	0.87 ± 0.60	0.47 ± 0.01
Phosphorus (P)	87.55 ± 18.85	83.64 ± 14.13	99.27 ± 33.50	77.05 ± 11.56	108.94 ± 19.82	97.26 ± 3.20
Zinc (Zn)	1.00 ± 0.31	1.02 ± 0.31	0.95 ± 0.42	0.78 ± 0.27	1.15 ± 0.14	1.34 ± 0.23
Aluminum (Al)	0.85 ± 0.39	0.89 ± 0.43	0.73 ± 0.30	0.93 ± 0.41	0.49 ± 0.04	1.18 ± 0.08
Manganese (Mn)	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020
Lead (Pb)	<0.015	<0.015	<0.020	<0.020	<0.015	<0.015
Arsenic (As)	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020
Boron (B)	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040
Barium (Ba)	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020
Cobalt (Co)	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020
Molybdenum (Mo)	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020
Nickel (Ni)	<0.020	<0.020	<0.040	<0.040	<0.020	<0.020
Selenium (Se)	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020
Strontium (Sr)	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Vanadium (V)	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010

Table 4.4: Microsoft Excel correlation matrix of giraffe milk and serum metabolites with giraffe milk nutrients

	Whey	*Casein*	*NPN*	*Total protein*	*Ca*	*Fe*	*P*	*Lactose*	*% Fat*	*Butyric acid*	*Caproic acid*	*Myristoleic acid*	*Palmitic acid*	*Linoleic acid*	*α-Linolenic acid*	*Conjugated linoleic acid*	*Arachidonic acid*
2-Ketoglutaric acid (milk metabolite)	0.46	-0.29	0.67	-0.16	0.59	0.83	-0.48	0.84	-0.05	0.76	0.81	-0.67	-0.78	0.77	-0.80	-0.78	-0.51
3-Hydroxybutyric acid (milk metabolite)	0.36	0.30	-0.50	0.43	-0.64	-0.50	0.12	-0.74	-0.28	-0.63	-0.70	-0.12	0.20	-0.50	0.63	0.72	0.48
Acetic acid (milk metabolite)	0.52	0.13	-0.33	0.31	-0.67	-0.34	-0.07	-0.59	-0.25	-0.57	-0.66	-0.23	0.09	-0.33	0.46	0.59	0.37
Acetone (milk metabolite)	0.09	0.48	-0.61	0.54	-0.73	-0.63	0.05	-0.82	-0.23	-0.48	-0.47	0.07	0.50	-0.65	0.56	0.83	0.32
Alanine (milk metabolite)	0.78	-0.48	0.40	-0.25	-0.24	0.56	-0.52	0.15	-0.37	0.18	0.04	-0.48	-0.55	0.42	-0.19	-0.25	0.06
Betaine (milk metabolite)	0.42	-0.29	0.61	-0.17	0.78	0.81	-0.19	0.61	-0.11	0.81	0.75	-0.57	-0.90	0.74	-0.50	-0.78	-0.26
Butyric acid (milk metabolite)	0.36	-0.77	0.61	-0.70	-0.36	0.57	-0.76	0.63	0.03	0.08	0.04	-0.09	-0.16	0.51	-0.65	-0.46	-0.34
Caproic/Caprylic acid (milk metabolite)	0.36	-0.77	0.59	-0.70	-0.39	0.55	-0.76	0.60	0.02	0.05	0.01	-0.07	-0.14	0.49	-0.62	-0.43	-0.32
Citric acid (milk metabolite)	0.13	0.03	0.42	0.07	0.43	0.39	-0.42	0.65	0.26	0.57	0.72	-0.43	-0.29	0.46	-0.75	-0.46	-0.69
Creatine (milk metabolite)	0.11	0.03	0.45	0.06	0.70	0.58	-0.26	0.58	0.03	0.88	0.97	-0.44	-0.53	0.54	-0.62	-0.60	-0.51
Creatinine (milk metabolite)	0.27	0.07	0.41	0.16	0.82	0.61	-0.11	0.60	-0.09	0.79	0.87	-0.63	-0.74	0.56	-0.53	-0.62	-0.36
Formic acid (milk metabolite)	0.94	-0.33	0.43	-0.04	-0.09	0.48	-0.56	0.14	-0.09	0.19	0.04	-0.72	-0.64	0.49	-0.27	-0.22	-0.16
Fumaric acid (milk metabolite)	0.17	0.04	0.31	0.10	0.25	0.29	-0.39	0.62	0.18	0.31	0.48	-0.45	-0.22	0.35	-0.67	-0.33	-0.57
Glycerophosphocholine (milk metabolite)	0.37	-0.34	0.67	-0.24	0.73	0.88	-0.31	0.74	-0.12	0.87	0.85	-0.53	-0.84	0.77	-0.64	-0.84	-0.35
Hippuric acid (milk metabolite)	0.53	-0.46	0.75	-0.31	0.45	0.91	-0.59	0.83	-0.11	0.76	0.77	-0.61	-0.78	0.81	-0.80	-0.80	-0.47
Isoleucine (milk metabolite)	0.59	0.06	-0.25	0.25	-0.66	-0.25	-0.15	-0.52	-0.25	-0.52	-0.61	-0.28	0.02	-0.24	0.38	0.51	0.32
Lactic acid (milk metabolite)	0.61	-0.07	-0.18	0.13	-0.70	-0.16	-0.21	-0.43	-0.30	-0.51	-0.62	-0.26	-0.01	-0.19	0.33	0.44	0.34
Lactose (milk metabolite)	-0.01	-0.44	0.72	-0.47	0.56	0.67	-0.44	0.91	0.38	0.66	0.71	-0.16	-0.38	0.72	-0.87	-0.82	-0.69
Leucine (milk metabolite)	0.71	-0.34	0.18	-0.13	-0.60	0.05	-0.51	-0.19	0.07	-0.33	-0.50	-0.30	-0.11	0.14	0.00	0.16	-0.03
N-Acetylgalactosamine (milk metabolite)	-0.29	0.10	0.32	0.01	0.56	0.22	-0.18	0.53	0.45	0.60	0.73	-0.07	-0.05	0.33	-0.62	-0.43	-0.68
N-Acetylglucosamine (milk metabolite)	0.18	-0.33	0.67	-0.29	0.81	0.72	-0.22	0.53	0.23	0.90	0.79	-0.29	-0.69	0.74	-0.52	-0.80	-0.44
Niacinamide (milk metabolite)	0.47	-0.27	0.64	-0.13	0.28	0.67	-0.70	0.75	0.12	0.63	0.70	-0.61	-0.52	0.68	-0.87	-0.60	-0.68
Orotic acid (milk metabolite)	0.10	-0.15	0.54	-0.12	0.66	0.68	-0.31	0.54	0.03	0.96	0.98	-0.31	-0.54	0.60	-0.60	-0.67	-0.47
Phenylalanine (milk metabolite)	0.53	0.19	-0.29	0.38	-0.68	-0.34	-0.20	-0.58	-0.15	-0.46	-0.52	-0.28	0.13	-0.30	0.34	0.59	0.18
Phosphocholine (milk metabolite)	0.09	-0.29	0.69	-0.28	0.63	0.63	-0.46	0.85	0.41	0.72	0.78	-0.33	-0.44	0.72	-0.88	-0.77	-0.77

	Whey	*Casein*	*NPN*	*Total protein*	*Ca*	*Fe*	*P*	*Lactose*	*% Fat*	*Butyric acid*	*Caproic acid*	*Myristoleic acid*	*Palmitic acid*	*Linoleic acid*	*α-Linolenic acid*	*Conjugated linoleic acid*	*Arachidonic acid*
Pyruvic acid (milk metabolite)	0.61	-0.38	0.64	-0.20	0.10	0.57	-0.74	0.75	0.22	0.31	0.36	-0.64	-0.48	0.66	-0.83	-0.52	-0.65
Succinic acid (milk metabolite)	0.46	-0.30	0.66	-0.17	0.40	0.78	-0.61	0.75	-0.03	0.77	0.82	-0.60	-0.64	0.71	-0.81	-0.69	-0.57
Taurine (milk metabolite)	0.33	-0.23	0.52	-0.13	0.70	0.76	-0.17	0.49	-0.20	0.84	0.79	-0.47	-0.80	0.63	-0.41	-0.69	-0.19
UDP (milk metabolite)	0.25	0.11	0.36	0.20	0.54	0.52	-0.32	0.48	-0.08	0.80	0.90	-0.55	-0.52	0.46	-0.57	-0.46	-0.46
UDP-Galactose (milk metabolite)	0.29	-0.13	0.47	-0.05	0.71	0.69	-0.14	0.40	-0.15	0.86	0.82	-0.45	-0.74	0.58	-0.37	-0.62	-0.22
UDP-Glucose (milk metabolite)	0.34	-0.20	0.56	-0.10	0.74	0.77	-0.22	0.56	-0.13	0.89	0.86	-0.52	-0.80	0.67	-0.51	-0.72	-0.30
UDP-N-Acetylgalactosamine (milk metabolite)	0.31	-0.22	0.48	-0.13	0.59	0.67	-0.18	0.31	-0.14	0.81	0.71	-0.37	-0.70	0.57	-0.30	-0.59	-0.17
UDP-N-Acetylglucosamine (milk metabolite)	0.23	-0.09	0.38	-0.02	0.59	0.58	-0.12	0.23	-0.15	0.82	0.75	-0.33	-0.61	0.47	-0.25	-0.50	-0.16
Uridine (milk metabolite)	0.51	-0.42	0.80	-0.27	0.65	0.91	-0.53	0.83	0.07	0.85	0.83	-0.64	-0.84	0.88	-0.82	-0.87	-0.56
Valine (milk metabolite)	0.58	0.13	-0.30	0.33	-0.58	-0.26	-0.06	-0.56	-0.31	-0.48	-0.58	-0.32	-0.03	-0.27	0.44	0.53	0.38
Serum 3-Hydroxybutyric acid	-0.09	0.35	-0.40	0.34	-0.56	-0.28	-0.10	-0.39	-0.40	-0.09	0.03	0.11	0.42	-0.46	0.19	0.50	0.16
Serum 3-Hydroxyisobutyric acid	-0.33	0.49	-0.42	0.41	-0.46	-0.50	-0.07	-0.36	0.02	-0.14	0.02	0.23	0.65	-0.50	0.09	0.54	-0.12
Serum Acetic acid	-0.10	0.29	-0.35	0.27	-0.63	-0.34	-0.20	-0.25	-0.21	-0.24	-0.09	0.12	0.52	-0.43	0.05	0.49	0.01
Serum Acetone	-0.33	0.73	-0.70	0.66	-0.41	-0.63	0.25	-0.59	-0.29	-0.26	-0.08	0.18	0.59	-0.72	0.43	0.73	0.24
Serum Alanine	0.05	0.08	-0.25	0.10	-0.42	0.00	-0.03	-0.38	-0.62	0.05	0.05	0.11	0.11	-0.28	0.31	0.29	0.40
Serum Allantoin	-0.23	0.57	-0.79	0.53	-0.68	-0.66	0.30	-0.70	-0.52	-0.54	-0.41	0.25	0.61	-0.82	0.62	0.84	0.54
Serum alpha-Glucose	0.51	-0.29	-0.11	-0.14	-0.68	0.02	-0.15	-0.26	-0.52	-0.51	-0.62	-0.12	-0.08	-0.13	0.32	0.28	0.52
Serum beta-Glucose	0.52	-0.36	-0.06	-0.21	-0.72	0.05	-0.22	-0.18	-0.48	-0.52	-0.62	-0.11	-0.06	-0.09	0.24	0.24	0.46
Serum Betaine	-0.18	0.60	-0.62	0.58	-0.47	-0.48	0.16	-0.41	-0.45	-0.30	-0.10	0.05	0.46	-0.63	0.32	0.64	0.28
Serum Citric acid	-0.36	0.67	-0.79	0.59	-0.57	-0.71	0.32	-0.70	-0.38	-0.43	-0.28	0.31	0.68	-0.83	0.58	0.83	0.41
Serum Creatine	-0.23	0.25	-0.15	0.19	-0.04	0.05	-0.04	-0.12	-0.36	0.42	0.52	0.12	0.14	-0.17	0.00	0.08	0.03
Serum Creatinine	-0.16	0.51	-0.71	0.49	-0.63	-0.54	0.26	-0.70	-0.59	-0.41	-0.31	0.21	0.50	-0.74	0.62	0.77	0.56
Serum Dimethyl sulfone	-0.01	0.20	-0.60	0.21	-0.76	-0.65	0.28	-0.82	-0.22	-0.79	-0.87	0.32	0.51	-0.66	0.77	0.78	0.60
Serum Formic acid	-0.65	0.59	-0.90	0.41	-0.31	-0.71	0.82	-0.66	-0.57	-0.58	-0.45	0.55	0.61	-0.91	0.85	0.72	0.82
Serum Fumaric acid	0.45	-0.52	0.09	-0.39	-0.51	0.26	-0.19	-0.06	-0.52	-0.29	-0.44	-0.05	-0.21	0.07	0.18	0.00	0.48
Serum Glutamic acid	0.06	0.10	-0.21	0.12	-0.56	-0.03	-0.24	-0.20	-0.51	-0.01	0.07	0.04	0.23	-0.27	0.07	0.30	0.18
Serum Glutamine	-0.14	0.38	-0.45	0.36	-0.50	-0.28	0.00	-0.26	-0.50	-0.18	0.00	0.08	0.39	-0.49	0.18	0.47	0.23
Serum Hippuric acid	-0.12	-0.25	-0.01	-0.30	-0.77	-0.14	-0.45	-0.01	0.04	-0.31	-0.26	0.36	0.56	-0.18	-0.16	0.23	-0.13

	Whey	*Casein*	*NPN*	*Total protein*	*Ca*	*Fe*	*P*	*Lactose*	*% Fat*	*Butyric acid*	*Caproic acid*	*Myristoleic acid*	*Palmitic acid*	*Linoleic acid*	*α-Linolenic acid*	*Conjugated linoleic acid*	*Arachidonic acid*
Serum Histidine	-0.29	0.43	-0.52	0.36	-0.53	-0.41	0.06	-0.47	-0.39	-0.14	-0.01	0.27	0.55	-0.58	0.31	0.57	0.24
Serum Isoleucine	-0.10	0.51	-0.55	0.50	-0.46	-0.38	0.12	-0.59	-0.49	-0.10	-0.01	0.10	0.37	-0.57	0.43	0.60	0.34
Serum Lactic acid	0.38	-0.45	0.04	-0.35	-0.42	0.25	-0.07	-0.11	-0.59	-0.23	-0.38	-0.01	-0.23	0.03	0.26	0.01	0.56
Serum Leucine	0.06	0.40	-0.56	0.45	-0.54	-0.40	0.18	-0.75	-0.52	-0.26	-0.27	0.07	0.28	-0.57	0.62	0.66	0.52
Serum Mannose	0.02	0.50	-0.48	0.54	-0.07	-0.21	0.34	-0.58	-0.59	0.07	0.09	-0.08	0.00	-0.41	0.54	0.42	0.49
Serum myo-Inositol	-0.36	0.45	-0.78	0.36	-0.74	-0.69	0.34	-0.77	-0.46	-0.58	-0.50	0.48	0.72	-0.85	0.70	0.84	0.60
Serum N,N-Dimethylglycine	-0.87	0.38	-0.59	0.12	-0.31	-0.75	0.48	-0.43	0.17	-0.51	-0.39	0.80	0.90	-0.70	0.44	0.55	0.21
Serum Phenylalanine	-0.20	0.43	-0.50	0.39	-0.38	-0.29	0.14	-0.50	-0.54	-0.01	0.09	0.19	0.35	-0.52	0.38	0.50	0.35
Serum Pyruvic acid	0.29	0.11	-0.50	0.22	-0.71	-0.35	0.18	-0.63	-0.58	-0.65	-0.72	0.01	0.17	-0.50	0.66	0.63	0.71
Serum Succinic acid	0.11	-0.36	-0.03	-0.34	-0.62	0.18	-0.23	-0.11	-0.59	-0.12	-0.16	0.22	0.10	-0.11	0.13	0.10	0.39
Serum Threonine	0.09	-0.11	-0.30	-0.08	-0.94	-0.26	-0.21	-0.32	-0.40	-0.57	-0.55	0.22	0.45	-0.41	0.25	0.49	0.35
Serum Tyrosine	-0.01	0.36	-0.66	0.38	-0.77	-0.51	0.18	-0.71	-0.59	-0.53	-0.49	0.18	0.46	-0.70	0.63	0.77	0.60
Serum Valine	-0.20	0.67	-0.70	0.64	-0.48	-0.59	0.24	-0.72	-0.39	-0.25	-0.13	0.15	0.52	-0.72	0.54	0.76	0.35

From Table B5 it is derived that myristoleic acid is the only metabolite to be significantly different for pregnancy status. NPN and arachidonic acid are the only metabolites to be significantly different for year of sampling. According to Table 4.4, the milk metabolome is more correlated with the nutrient concentrations than the serum metabolome.

4.4. Discussion

The following sections discuss the variation in the nutrient concentrations between the groups of giraffes as well as the variation between species.

4.4.1. Variation of nutrient concentrations

Different species have different nutrient requirements (Oftedal et al., 1995; Berdanier et al., 2007; Oftedal, 2012;) and an example of the variation in milk nutrients between different species was shown in Table 1.1. The concentrations of milk nutritents differs between the ruminants (giraffe, cow, and goat), carnivores (cheetah and panda), and non-ruminants (donkey).

The milk nutrient content in the milk of the giraffe as well as the common domestic ruminants, cow and goat is shown in Table B8. Although the giraffe is a ruminant, the nutrient composition differs from that of cow and goat. There is a higher amount of fat in the milk of the giraffe when compared to that of the cow and goat. There is a lower concentration of butyric acid in the milk fat of the giraffe when compared to that of the cow and goat. Milk of ruminants have a higher amount of these short chain fatty acids as they are produced by the rumen bacteria (Chesworth et al., 1998; Christie, 2014). However, the giraffe is a ruminant and literature (Osthoff et al., 2017) has also reported a lower butyric fatty acid concentration for giraffes and this may suggest an individual species trend for these ruminants. The rumen bacteria and microbes may also differ (Mizrahi, 2013).

4.4.3. Milk nutrients of giraffes in different locations

The nutrient composition of giraffe milk has been reported previously (Osthoff et al., 2017). These giraffes were located at the Khamab Kalahari Reserve, Molopo district of the Eastern Kalahari Bushveld, as well as the Amanzi Private Game Reserve, Brandfort. The milk in that study was collected in February, the rainy season, when the forage was in excellent condition

(F. Deacon, personal communication). This difference in environmental conditions and nutrition, may explain the differences in milk nutrients between the 2017 report and the current one. The fat content in the milk of these giraffes was reported to be higher than that of cows and goats. The fat content in the milk of the giraffes located in Sanveld and Rooiport reserve are also high when compared to these domestic ruminants. It is noted that there might be differences in contents of parameters according to location, but no statistical evidence is possible due to the small number of individuals.

The milk of only one non-pregnant lactating giraffe was obtained from Sandveld reserve. The date of sampling of the giraffe located in Sandveld reserve and the quality of browse were different from that of giraffes located in Rooiport reserve. In both 2017 and 2018, Rooiport reserve was experiencing a drought (see weather data in appendix C). Sandveld giraffe milk contains a higher total protein, capric acid, myristic acid, vaccenic acid, saturated fatty acid and aluminium content. Sandveld giraffe milk is also lower in oleic acid, mono-unsaturated fatty acids, sodium, potassium and iron.

4.4.4. Factors affecting the nutrient content of giraffe milk

The fat content of giraffe milk reported in literature (Osthoff et al., 2017) is higher than the giraffe average obtained here. This may be because the giraffes in that study were sampled in the middle of summer when browsing material was of high quality (see comment above). The giraffes for the study in this thesis were sampled during a season of drought and this affected the forage (see Appendix C for more information on the weather data). Dietary changes such as plant matter with a higher roughage content versus plant matter with a lower roughage content can cause changes in the nutrient composition of ruminant milk (Orskov, 1986; Sutton et al., 2003).

Diet significantly affects the concentration of fatty acids in milk (Rego et al., 2016; Woods & Fearon, 2009; Mackle et al., 1999; Chilliard et al., 2001; Nantapo et al., 2014). Dietary fat contributes to the fatty acid composition of milk and factors such as lactation stage and diet can cause variation in the milk fatty acid composition (Iverson & Oftedal, 1995). High fibre can decrease intake and digestibility (Council, 2001). Ruminant digestion of fibre results in the production of acetic acid. The higher the fibre content, the higher the acetic acid production. Acetic acid is the major precursor of fat in the mammary gland (Perry, 1980; J. Moran, 2005). Reduction in fibre leads to a reduction in acetic acid which is then seen as a reduction of milk fat in the milk.

Although differences were noted in the fat content and mono-unsaturated fatty acid content in milk of the pregnant lactating and non-pregnant lactating giraffes, the differences were not significant (see table B5). These differences may be a result of dietary changes. In a study on dairy cows, reduced energy intake caused differences in fatty acid profiles (Mackle et al., 1999).

The only milk compound that differs significantly between pregnant lactating and non-pregnant lactating giraffes is myristoleic acid ($p=0.0429$). The enzyme Δ^9-desaturase is involved with unsaturated fatty acid biosynthesis and the only source for myristoleic acid in milk fat is desaturation of myristic acid by this enzyme. Myristic acid originates from mammary gland synthesis (Griinari et al., 2000).

The only milk compound that differs significantly between year of sampling is arachidonic acid ($p=0.0445$). Arachidonic acid is obtained from diet or by desaturation and chain elongation of linoleic acid (Tallima & El Ridi, 2018).

Milk compounds that differ between the 2017 and 2018 giraffe groups are fatty acids. Arachidonic acid is the only one differing statistically at $p<0.05$. Many other fatty acids differed at $p<0.0507$ and $p<0.0528$. This might be the result of dietary changes as discussed in studies on dairy cows (Chilliard et al., 2001; Woods & Fearon, 2009) and goats (Iussig et al., 2015). Only a study with larger number of subjects and a thorough nutritional study design would clarify this.

In a study on the influence of nutritional factors on cow's milk, different forages were said to affect cow milk fat composition (Chilliard et al., 2001). Forages with higher fibre have a lower fatty acid content (Glasser et al., 2013) and dietary fatty acids are transferred into milk (Woods & Fearon, 2009). In that study on dairy cows (Woods & Fearon, 2009), the milk linoleic acid content was higher for cows on a diet of fresh grass and lower for cows fed forages such as hay which have a higher fibre content. The amount of linoleic acid in fresh grass also differs between seasons (Chilliard et al., 2001). The fatty acid content of giraffe milk may be influenced by the botanical composition of the ingested browsed forages due to the variability of unsaturated fatty acid levels in these forages. In a study on browsing ratio and plant species intake and its effect on goat milk, the fatty acid profile of the ingested plants altered the fatty acid profile of goat milk. The ingested vegetation contained woody species and herbs which were positively associated with the nutritionally desirable fatty acids in goat milk (Iussig et al., 2015).

Fatty acids such as linoleic acid are present in higher concentrations for the 2017 giraffe group (2.59 ± 0.27 % of total) when compared to the 2018 group (0.23 ± 0.06 % of total). Linoleic acid is also present in higher concentrations in the milk of the non-pregnant giraffe group (1.92

± 1.17 % of total) when compared to the pregnant giraffe group (1.33 ± 1.64 % of total). Stage of lactation was said to affect fatty acid profiles of dairy cows (Nantapo et al., 2014) and linoleic acid was shown to be highest in mid lactation. This also coincided with the pastures having the highest amount of linoleic acid. The lactation time for the giraffes was not determined but it was estimated to be between 2.5 and 4.5 months and the different fatty acid profiles between the different groups may be related to factors such as diet and the stage of lactation.

There is no significant difference in giraffe milk lactose concentration between the pregnant and non-pregnant groups. There is a lower lactose concentration in the 2018 group when compared to the 2017 group, however, according to Table B13 this is not significant. This confirms the results obtained by NMR in Chapter 3. A lower roughage diet with a higher energy density produces high amounts of propionic acid (Orskov, 1986). Glucose in ruminants is primarily obtained from gluconeogenesis in the liver using propionic acid. Blood glucose is used for the synthesis of lactose (Frandson et al., 2009). The drop in lactose content for the 2018 giraffe group may be an indication that the 2017 group of giraffes ate enough plant material with a lower fibre content and higher energy density to have a sufficient glucose supply to produce a good amount of lactose.

The NPN content in the milk of the 2018 giraffe group (0.10 ± 0.02 g/100g) is lower than the 2017 giraffe group (0.17 ± 0.03 g/100g) and this is a significant difference ($p = 0.0424$). This suggests that the 2018 giraffe group had access to plant material containing a higher amount of protein and lower NPN content. In a study on the NPN content in the milk of dairy cows (Ruska & Jonkus, 2015), the NPN content was significantly affected by different housing and feeding technologies. Increased milk protein and NPN levels in the milk of dairy cows is due to a diet containing high levels of rumen-degradable protein or NPN (Linn, 1988).

In studies on the effect of dietary protein on cow's milk, it was shown that a diet deficient in protein reduces the milk protein content (Schingoethe, 1996; M'hamed et al., 2001). As discussed in section 2.4.2., year 2018 received less rainfall than 2017 and this had a detrimental effect on the plants, specifically the trees that the giraffes browsed. The nutrient deprived browsing may be responsible for the change in NPN concentrations. Giraffes favour the leaves of acacia trees (Parker, 2005), but, due to the limited amount of leaves available in 2018, perhaps the giraffes ate different parts of the plant. Nutritional assessment of *Acacia catechu willd* showed that the stems, pods, and bark all contained varying nutrient concentrations (Verma et al., 2014).

The calcium content of pregnant giraffe milk is not significantly higher when compared to the non-pregnant giraffes and the Sandveld giraffe. Iron is particularly low for the 2018 giraffe group (0.87 ± 0.60 g/100g) when compared to the 2017 giraffe group (6.21 ± 1.84 g/100g). This difference was not significant (p=0.0528). A clearer indication of the significance, can be achieved by increased sample size. Giraffes favour the leaves of the Acacia tree (Parker, 2005) and the iron content in the leaves varies between species (Abdulrazak et al., 2000; Derero & Kitaw, 2018) and different parts of the plant (Verma et al., 2014). Diet can be responsible for changes in mineral content in milk (Al-Wabel, 2008; Linn, 1988). The lower iron content in the 2018 giraffe samples may be due to a dietary change between the two years which is then reflective in the milk.

4.4.5. Correlation of serum and milk metabolites with milk nutrients

Pearson correlation coefficients were calculated using Excel 2016 (Microsoft, Redmond, Washington) and the correlation table (Table 4.4) shows a correlation of the giraffe milk and serum metabolites with the giraffe milk nutrients. According to this table, milk metabolites were more strongly correlated with milk nutrients than serum metabolites. There are many correlations between singular parameters, but the focus will be on the group-effects of metabolites on milk nutrients.

The mammary gland secretory cells synthesize milk proteins using amino acids from the blood (Frandson et al., 2009). The serum metabolites isoleucine and valine are positively correlated with milk total protein and casein protein. The other serum amino acids present are only weakly correlated with milk total protein. The milk metabolites alanine, isoleucine, leucine, phenylalanine, and valine are positively correlated with milk whey protein. There is a weak correlation of these same amino acids with milk total protein. In a study on milk metabolites and milk traits of Holstein cows, asparagine and aspartic acid were positively correlated with casein and total protein content (Melzer et al., 2013). However, asparagine and aspartic acid in giraffe milk were likely below the levels of detection and were not present in the giraffe milk metabolome (Emwas, 2015).

The serum amino acids isoleucine, leucine, phenylalanine, tyrosine, and valine are all negatively correlated with milk lactose. The concentrations of these amino acids are higher for the 2018 group and the milk lactose concentration is lower for the 2018 group when compared to the 2017 group. The low energy intake for the 2018 group is responsible for the lower

lactose concentration as discussed in section 3.4.1. and this is correlated with the higher concentrations of serum amino acids because serum amino acids, specifically branched chain amino acids (BCAAs), increase in concentrations during starvation (Adibi, 1976; Felig et al., 1969).

α-lactalbumin is an important protein involved in the synthesis of lactose and amino acids are precursors of these proteins (Melzer et al., 2013; Brew et al., 1968). The milk and serum metabolites phenylalanine, isoleucine, and valine were all negatively correlated with the milk nutrient lactose which suggests that these amino acids are important for α-lactalbumin synthesis. Phenylalanine and valine are glucogenic amino acids (Campbell & Farrell, 2012) and may have been used to produce glucose which was then used for lactose production (Frandson et al., 2009). The same milk amino acids are positively correlated with the whey proteins, which include α-lactalbumin. Together this could be interpreted that α-lactalbumin production is promoted by these amino acids, as well as the synthesis of glucose and lactose. It would be interesting to study this simultaneous effect specifically on α-lactalbumin, and not just the total whey proteins. The concentrations of the mentioned amino acids in milk and serum are lower for the 2017 giraffe group when compared to the 2018 giraffe group and this corresponds with the higher amount of lactose for the 2017 giraffe group. Isoleucine and valine are BCAAs and their concentrations increase in the blood during starvation. BCAA concentrations are more prominently affected than other amino acids (Adibi, 1976). There may be a spill over of these amino acids into the milk and their positive correlation with the whey proteins may indicate active whey protein synthesis.

Giraffe milk fat precursors are not significantly correlated with giraffe milk fat. Acetic acid is the major precursor of fat in the mammary gland (Perry, 1980; J. Moran, 2005). 3HBA is also a precursor of milk fat (Barbosa-Cánovas et al., 2006). Giraffe serum acetic acid ($p = -0.21$), serum 3HBA ($p = -0.40$), milk acetic acid ($p = -0.25$) and milk 3HBA ($p = -0.28$) show a weak negative correlation to milk fat.

Calcium citrate and calcium phosphate compose most of the aqueous calcium in bovine milk. (Neville et al., 1994). Literature showed that citrate is positively correlated with calcium in sows' milk (Kent et al., 1998). Giraffe serum citric acid is negatively correlated with milk calcium. There is a weak positive correlation between the giraffe milk metabolite citrate with giraffe milk calcium.

In a study on the milk metabolomics of Brown Swiss and Simmental cows, a positive correlation was observed between N-acetylcarbohydrates and total protein content in milk. The correlation was attributed to the presence of acetyllactosamine in many glycoproteins and

glycolipids (Klein et al., 2010). Giraffe milk shows a very weak correlation of N-Acetylgalactosamine (p = 0.01) and N-Acetylglucosamine (p = -0.29) with milk total protein.

The giraffe milk metabolites creatine and creatinine show positive correlations with giraffe milk calcium, iron, lactose, butyric acid, caproic acid, and linoleic acid. In a study on creatine and creatinine phosphate in sow milk, a positive correlation was seen between sow milk creatine and lactose (Probert et al., 1997) and it was concluded that creatine and creatine phosphate concentrations might reflect mammary gland metabolic activity. Giraffe milk creatine, creatinine, calcium, iron, lactose, butyric acid, caproic acid, and linoleic acid decreased in concentration for the 2018 giraffe group when compared to the 2017 giraffe group. The decrease may be due to the giraffes being on a lower plain of nutrition in 2018 because of the lower amount of rainfall received for that year. Barbosa et al. (2014) reported that leaf nutrient content depends on water availability. The correlation of creatine and creatinine with calcium, iron, lactose, butyric acid, caproic acid, and linoleic acid are likely related to them all being influenced by the change in nutrient availability from the giraffes' diet. Changes in the mineral concentrations (Al-Wabel, 2008; Linn, 1988) and fatty acid concentrations (Rego et al., 2016; Woods & Fearon, 2009; Chilliard et al., 2001) are affected by changes in diet. Lactose concentrations in milk are affected by diet as discussed in section 3.4.1. Intake of protein affects the excretion of creatine in the urine (Catherwood & Stearns, 1937; Denis, 1917) and it might also affect the excretion of creatine in milk. Creatine is taken up into the blood in order to be excreted (MacNeil et al., 2005) and Lamarre et al., (2010) concluded that the rat mammary gland extracts creatine from the blood rather than synthesizing it. Creatinine concentrations are also affected by diet. Lamp et al. (2015) reported that the levels of creatinine in serum are influenced by protein intake. Metabolites from the blood are taken up by the mammary gland for milk synthesis (Frandson et al. 2009; Davis & Collier, 1985) and the concentrations of creatine and creatinine as well as calcium, iron, lactose, butyric acid, caproic acid, and linoleic acid in the giraffe milk may all have been influenced by the quality of nutrition received and is responsible for their correlation.

The giraffe milk metabolites N-Acetylglucosamine, UDP-N-Acetylglucosamine, UDP-galactose, and UDP-glucose which are part of the amino sugar and nucleotide sugar metabolism (www.metaboanalyst.ca) as well as orotic acid, UDP and uridine, which are part of the pyrimidine metabolic pathway (www.metaboanalyst.ca), are also all positively correlated with calcium, iron, lactose, butyric acid, caproic acid, and linoleic acid. UDP-N-Acetylglucosamine, however, shows a weak positive correlation with lactose. As with creatine and creatinine mentioned above, these correlations may also be because of diet.

The milk metabolites UDP-glucose and UDP-galactose are part of the galactose metabolic pathway (www.metaboanalyst.ca) and are positively correlated with milk lactose. These two metabolites are involved with lactose synthesis (Anderson et al., 2014). Lactose is synthesized inside the secretory cells of the mammary gland. These cells synthesize galactose by using glucose from the blood. The galactose is then combined with glucose to form lactose (Frandson et. al 2009). Milk 2-ketoglutaric acid, citric acid, pyruvic acid, succinic acid, and fumaric acid are all part of the citric acid cycle (www.metaboanalyst.ca). These metabolites are also positively correlated with lactose. In a study on human milk, lactose levels were positively correlated to citric acid and 2-ketoglutaric acid (Gay et al., 2018). The citric acid cycle is the main pathway of carbohydrate metabolism and supplies energy to all living cells (Grassian et al., 2014). As suggested in section 3.4.1., a higher production of propionate due to a lower roughage diet would lead to an increase in glucose production. More glucose would be available to enter the citric acid cycle and there would be an increase in the citric acid cycle related metabolites. Glucose is also needed for the synthesis of lactose (Frandson et al., 2009) and this may be why the citric acid cycle metabolites are positively correlated with lactose.

Serum alanine, citric acid, fumaric acid, succinic acid, pyruvic acid, glutamic acid, and glutamine are all part of the alanine, aspartate and glutamate metabolic pathway (www.metaboanalyst.ca). These metabolites show a negative correlation with giraffe milk calcium and fat. In a study on the metabolomic profiles in yak (*Bos grunniens*) mammary gland tissue, the alanine, aspartate and glutamate metabolism was one of the most impacted pathways during the lactation cycle. Perhaps change of diet could have also impacted this pathway for giraffes. Intraruminal infusions of amino acids (Schwab et al., 1976) have had no effect on milk fat percentage in cows. The concentrations of fat and calcium for the 2018 giraffe group are lower when compared to the 2017 giraffe group. Serum alanine, citric acid, fumaric acid, succinic acid, pyruvic acid, glutamic acid, and glutamine are higher in concentration for the 2018 giraffe group. The alanine, aspartate and glutamate metabolic pathway may have been more active for the 2018 group. The lower quality of nutrition for the 2018 group may have caused this and may also be responsible for the lower concentrations of calcium and fat.

Other correlations include serum amino acids with fatty acids, lactose and calcium as well as serum acetate, allantoin, dimethyl sulfone, formic acid, and inositol with linoleic acid, NPN and lactose. The milk metabolites alanine, phenylalanine, valine, isoleucine, and leucine are involved with aminoacyl-tRNA biosynthesis (www.metaboanalyst.ca) and are positively correlated with whey protein. Serum threonine, pyruvic acid, N,N-Dimethylglycine, creatine, and betaine are part of the glycine, serine and threonine metabolic pathway (www.metaboanalyst.ca). These metabolites don’t show any group correlations with the milk

nutrients. Serum lactate, pyruvate, acetic acid, and fumaric acid are part of the pyruvate metabolic pathway (www.metaboanalyst.ca) and there are no group correlations with any of the milk nutrients for these metabolites.

4.5. Conclusion

In this chapter, the milk nutrient content of non-pregnant lactating female and pregnant lactating female giraffes was determined. There were no significant differences in nutrients between lactating and non-lactating giraffes, and also not between milk samples collected in 2017 and 2018.The only significant differences noted were for myristoleic acid was significant ($p=0.0429$) for pregnancy status, and NPN and arachidonic acid for the sampling years. The serum amino acids isoleucine and valine were positively correlated with milk total protein and casein protein. The serum amino acids isoleucine, leucine, phenylalanine, tyrosine, and valine are all negatively correlated with milk lactose. Serum citric acid is negatively correlated with milk calcium. The milk metabolites alanine, isoleucine, leucine, phenylalanine, and valine are positively correlated with milk whey protein. The milk metabolites phenylalanine, isoleucine, and valine are negatively correlated with milk lactose. The milk metabolite citrate is positvely correlated with calcium. Diet may be responsible for the positive correlations of creatine and creatinine with some of the milk nutrients.

Chapter 5 – Concluding discussion

The giraffe is the largest ruminant and is one of very few species that can fall pregnant while simultaneously nursing a calf (Deacon et al., 2015). This allowed the pregnancy status of the female giraffes to be investigated as a variable. Blood and milk were collected from eleven female giraffes and blood from nine males. Blood and milk were also obtained from two females of a different reserve. The metabolites in serum and milk were analysed by nuclear magnetic resonance spectroscopy (NMR) and the milk nutrients (protein, carbohydrate, fat and fatty acid, and minerals) according to standard procedures. The aims were to obtain a baseline metabolome of giraffe serum, a baseline metabolome of giraffe milk, and to determine whether there is any obvious interrelationship between the metabolomes and milk nutrients.

The NMR serum metabolome of male, non-pregnant lactating female, and pregnant lactating female giraffes were determined in Chapter 2. There were significant sex differences for several metabolites in the giraffe serum, specifically pyruvic acid, citric acid, creatine, threonine, phenylalanine and glutamine. This showed that the alanine, aspartate, and glutamate metabolism, as well as the citric acid cycle, were more active in male giraffes. The metabolic demands of the female giraffes were greater due to lactation and pregnancy. A higher activity of these pathways was expected for the males. Gender differences of plasma metabolites have also been observed in humans (Trabado et al., 2017).

The significant differences of giraffe serum metabolomes between 2017 and 2018 may be because of environmental conditions of rainfall and average temperatures, which affected the forage and may have caused stress. Parameters that differed significantly in the serum were citric acid, betaine, creatinine, formic acid, acetone, pyruvic acid, myo-Inositol, glutamine, dimethyl sulfone, allantoin, fumaric acid, histidine, alanine, threonine, mannose, tyrosine, leucine, succinic acid, and phenylalanine were lower in 2017 than in 2018. This showed that the alanine, aspartate, and glutamate metabolism, as well as the citric acid cycle, were more active in the 2018 giraffe group. It was reasoned that the environmental stress might have caused higher cortisol levels which could have resulted in the catabolism of muscle and an increase in the circulating amino acids in blood (Thung & Norwitz, 2009). When the metabolomes of only female giraffes were compared, the number of differing metabolites was narrowed down to formic acid, citric acid and myo-inositol, which indicated to a higher activity

of the citric acid cycle. In a study on the metabolites in the four biofluids of Holstein dairy cows, the citric acid cycle was observed to be more active in the lactating group when compared to the non-lactating group due to nutritional stress experienced by the lactating group (Sun et al., 2017).

A comparison of the giraffe serum metabolome with the human serum metabolome (Psychogios et al., 2011) showed that alanine was present in much higher concentrations in human serum compared to giraffe serum, as was citric acid. The other citric acid cycle metabolites, fumaric acid and succinic acid, are either absent or present in levels below detection in human serum.

The milk metabolome of non-pregnant lactating and pregnant lactating giraffes was determined in Chapter 3. The milk contains a high concentration of lactose, as well as the metabolites that are involved in the lactose synthesis pathway. These are metabolites of the citric acid cycle, as well as UDP-glucose and UDP galactose (Anderson et al., 2014).

Metabolites from the blood are taken up by the mammary gland to be used for milk synthesis, and the mammary gland cells internally regulate the milk nutrients and their concentrations (Frandson et al., 2009). Pregnancy status and year did not significantly affect the milk metabolome. A larger number of subjects would be needed to substantiate this. However, there were a number of metabolites which significantly differed at $p = 0.0528$. Specifically to be mentioned are a lower lactose concentration in 2018, as well as metabolites of the lactose synthesis path, such as UDP-glucose and UDP-galactose. The differences between year 2017 and 2018 suggested that the giraffe milk metabolome was affected by a decreased level of nutrition, such as digestible carbohydrates which favoured the production of propionic acid (Solaiman, 2010) and in turn supplied them with sufficient glucose to produce a higher amount of lactose (Elsden, 1945; Orskov, 1986).

Comparison of giraffe milk metabolites with other species is not straight forward because different analytical methods used to analyse the metabolites may likely be responsible for the differences. Table A1 to A6 in the appendix shows the metabolites present in the milk of different species. In the study on Holstein cows, the GC-MS analyses measured only part of the milk metabolome (Melzer et al., 2013) and this is why some metabolites may be absent for Holstein cows when compared to the study done on the Brown Swiss and Simmental cows which used both NMR and GC-MS analyses (Klein et al., 2010).

There are differences in the giraffe serum and milk metabolomes. There are metabolites found in giraffe serum which are not found in giraffe milk and vice versa. The metabolites which are present in milk and not serum show that milk cells have a metabolism of their own. The most

obvious example is the production of lactose (Anderson et al., 2014; Frandson et al., 2009). Another example is glycerophospholipid synthesis. Phosphocholine and glycerophosphocholine are also present in giraffe milk and not giraffe blood. These two metabolites are involved in glycerophospholipid metabolism (Matsuda et al., 2017). In a study on the major choline metabolites in rat milk, rat mammary epithelial cells, in primary culture, synthesized and secreted phosphocholine and glycerophosphocholine (Rohlfs et al., 1993).

The milk nutrient content of non-pregnant and pregnant giraffes was documented in Chapter 4. NPN and arachidonic acid differed significantly over the two years. NPN showed a higher concentration in the milk of the 2017 group and arachidonic acid showed a higher concentration in the milk of the 2018 group. Several other fatty acids also differed which might be the result of dietary differences as was found in studies of dairy cows (Chilliard et al., 2001; Woods & Fearon, 2009) and goats (Iussig et al., 2015). The only milk compound that differed between pregnant and non-pregnant giraffes was myristoleic acid. Myristoleic acid was present in the milk of the pregnant giraffe group at higher concentrations than the non-pregnant group.

A change of diet may have influenced the concentration of the milk fat and lactose concentrations for the 2017 and 2018 group. Although not a significant difference, the fat content of the giraffes from the 2017 group was higher than that of the 2018 group. The lactose concentration was also higher for the 2017 giraffe group when compared to the 2018 group. These differences might also result from the effect of environmental conditions on the browse, as was mentioned above. The differences in the amount of rainfall received between year 2017 and 2018 may have affected the NPN and arachidonic acid concentrations found in plant matter which was then reflected in the milk. This suggestion may be supported by the findings of a study on the effect of water availability and leaf nutrient concentrations of savanna trees in South Africa, uneven water availability caused leaf nutrient concentrations to decrease. Regular rainfall caused an increase in leaf nutrient concentrations (Barbosa et al., 2014).

Nutrition affects the quality of cow milk and its components (Mackle et al., 1999; Jenkins & McGuire, 2006; Tyasi et al., 2015) and this may also be true for giraffe milk. The lower concentration of serum and milk metabolites for the pregnant lactating group, when compared to the non-pregnant lactating group suggest that pregnant lactating giraffes have higher energy demands. The milk nutrient concentrations of these two groups seemed to be quite similar and this may suggest that the mammary gland cells internally regulate the milk nutrients and their concentrations, regardless of metabolic concentration irregularities due to nutritional stress.

A correlation of the giraffe milk and serum metabolites with the giraffe milk nutrients showed that the milk metabolome has a greater effect on the nutrient concentrations than the serum metabolome. The milk and serum metabolites phenylalanine, isoleucine, and valine were all negatively correlated with the milk nutrient lactose. The concentrations of these amino acids in milk and serum are lower for the 2017 giraffe group when compared to the 2018 giraffe group and this corresponds with the higher amount of lactose for the 2017 giraffe group. The giraffe milk metabolite citrate is positvely correlated with calcium. Literature showed that citrate is also positively correlated with calcium in sows' milk (Kent et al., 1998). Diet may be responsible for the positive correlations of creatine and creatinine with some of the milk nutrients. Milk metabolites which are part of the amino sugar and nucleotide sugar metabolism and pyrimidine metabololism show positive correlations to some of the milk nutrients and diet may also be a factor here. Milk citric acid cycle metabolites are positively correlated with lactose. A lower roughage diet would lead to an increase in glucose production which would mean that there is more glucose available for lactose production (Frandson et al., 2009) as well as more glucose to enter the citric acid cycle.

It has to be kept in mind that the giraffe population of this study was small, and that the groupings into male, female, pregnant and non-pregnant and year created even smaller groups for comparison. This affected the statistical interpretation of the data, so that the data can only be seen as mere observation. Nevertheless, the data provide guidelines for future work, whether on giraffes or other species. Although it is not always possible in studies of wild animals that to include a proper experimental design, it should be strived that a larger group of animals be included and that the quality of nutrition be analysed and described.

Chapter 6 - References

Abdulrazak, S.A., Fujihara, T., Ondiek, J.K. & Érskov, E.R. (2000) Nutritive evaluation of some Acacia tree leaves from Kenya. *Animal Feed Science and Technology*, 85, pp.89–98.

Abu-Ghazaleh, A.A., Schingoethe, D.J. & Hippen, A.R. (2001) Blood Amino Acids and Milk Composition from Cows Fed Soybean Meal, Fish Meal, or Both. *Journal of Dairy Science*, 84 (5), pp.1174–1181.

Adewuyi, A.A. & Gruys, E. (2011) Non-esterified fatty acids (NEFA) in dairy cattle. A review. *Veterinary Quarterly*, 2176 (3), pp.117–126.

Adibi, S. A. (1976) Metabolism of branched-chain amino acids in altered nutrition. Metabolism: clinical and experimental, 25(11), pp. 1287–1302.

Al-Wabel, N.A. (2008) Mineral Contents of Milk of Cattle, Camels, Goats and Sheep in the Central Region of Saudi Arabia. *Asian Journal of Biochemistry*, 3 (6), pp.373–375.

Anderson, S.M., MacLean, P.S., McManaman, J.L. & Neville, M.C. (2014) Lactation and its Hormonal Control. In: *Knobil and Neill's Physiology of Reproduction: Two-Volume Set*. Elsevier Inc., pp.2055–2105.

Bagchi, D., Swaroop, A. & Bagchi, M. (2015) *Genomics, proteomics and metabolomics in nutraceuticals and functional foods*. Hoboken, John Wiley & Sons, Ltd.

Bajpai, P. (2014) Chemicals Used in Deinking and Their Function. *Recycling and Deinking of Recovered Paper*, pp.121–137.

Barbosa, E.R.M., Tomlinson, K.W., Carvalheiro, L.G., Kirkman, K., de Bie, S., Prins, H.H.T. & van Langevelde, F. (2014) Short-Term Effect of Nutrient Availability and Rainfall Distribution on Biomass Production and Leaf Nutrient Content of Savanna Tree Species B. Bond-Lamberty ed. *PLoS ONE*, 9 (3), pp.1-9

Barbosa-Cánovas, G. V, Hartel, R.W., Labbe, R.G., Lund, D.B., Min, D.B., Nollet, L.M.L., Steele, J.L., Whitaker, J.R. & Yada, R.Y. (2006) *Dairy Science and Technology*. New York, CRC Press.

Bayly, G.R. (2014) Lipids and disorders of lipoprotein metabolism. *Clinical Biochemistry: Metabolic and Clinical Aspects*, pp.702–736.

Berdanier, C.., Dwyer, J.T. & Feldman, E. (2007) *Handbook of Nutrition and Food*. Florida, Taylor & Francis Group, LLC.

Bezerra, L. R., Neto, C. B. de O., Araújo, M. J. de, Edvan, R. L., Oliveira, W. D. C. de, & Pereira, F. B. (2014). Major Metabolic Diseases Affecting Cows in Transition Period. *International Journal of Biology*, 6(3), 85–94.

Bhagavan, N.V., Ha, C.-E., Bhagavan, N.V. & Ha, C.-E. (2011) Protein and Amino Acid Metabolism. In: *Essentials of Medical Biochemistry*. Elsevier, pp.169–190.

Brew, K., T. C. Vanaman, and R. L. Hill. 1968. The role of alpha- lactalbumin and the A protein in lactose synthetase: A unique mechanism for the control of a biological reaction. Proc. Natl. Acad. Sci. USA 59:491–497.

Brosnan, J.T. (2003) Interorgan Amino Acid Transport and its Regulation. *The Journal of Nutrition*, 133 (6), pp.2068S-2072S.

Brown, D.L., Van Wettere, A.J. & Cullen, J.M. (2017) Hepatobiliary System and Exocrine Pancreas. *Pathologic Basis of Veterinary Disease*, pp.412-470.

Caboni, P., Murgia, A., Porcu, A., Demuru, M., Pulina, G., & Nudda, A. (2016). Gas chromatography-mass spectrometry metabolomics of goat milk with different polymorphism at the α S1 -casein genotype locus. *Journal of Dairy Science*, 99, 1–6.

Campbell, M.K. & Farrell, S.O. (2012) *Biochemistry*. 7th edn. Brooks/Cole Cengage Learning, Belmont, CA.

Casares, M., Bernhard, A., Gerique, C., Malo, E. & Carbonell, D. (2012) Hand-rearing Rothschild or Baringo giraffe Giraffa camelopardalis rothschildi calves at Bioparc Valencia, Spain, and Leipzig Zoo, Germany. *International Zoo Yearbook*, 46 (1), pp.221–231.

Castrogiovanni, P. & Imbesi, R. (2017) The role of malnutrition during pregnancy and its effects on brain and skeletal muscle postnatal development. *Journal of Functional Morphology and Kinesiology*, 2 (30), pp.1-10.

Catherwood, R. and Stearns, S. G. (1917) Creatine and creatinine excretion in infancy. The Journal of Biological Chemistry, 119, pp. 201-214.

Cavendish, M. (2010) *Mammal Anatomy: An Illustrated Guide*. New York, Marshall Cavendish Corporation.

Champe, P.C., Harvey, R.A. & Ferrier, D.R. (2005) *Biochemistry*. 3rd edn., Lippincott Williams & Wilkins, Philadelphia, PA.

Chesworth, J., Stuchbury, T. & Scaife, J. (1998) *An Introduction to Agricultural Biochemistry*. 1st ed. London, Chapman & Hall.

Chilliard, Y., Ferlay, A. & Doreau, M. (2001) Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livestock Production Science*, 70 (1–2), pp.31–48.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D.S. & Xia, J. (2018) MetaboAnalyst 4.0: Towards more transparent and integrative metabolomics analysis. *Nucleic Acids Research*, 46 (W1), pp.W486–W494.

Chong, J., Wishart, D.S. & Xia, J. (2019) Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Current Protocols in Bioinformatics*, 68 (1).

Chong, J. & Xia, J. (2018) MetaboAnalystR: an R package for flexible and reproducible analysis of metabolomics data. *Bioinformatics (Oxford, England)*, 34 (24), pp.4313–4314.

Chong, J., Yamamoto, M. & Xia, J. (2019) MetaboAnalystR 2.0: From raw spectra to biological insights. *Metabolites*, 9 (3).

Christie, W.W. (2014) *Lipid Metabolism in Ruminant Animals*. 1st ed. New York, Pergamon Press.

Contarini, G., & Povolo, M. (2013). Phospholipids in milk fat: Composition, biological and technological significance, and analytical strategies. International *Journal of Molecular Sciences*, 14(2), 2808–2831.

Council, N.R. (2001) *Nutrient Requirements of Dairy Cattle.* Washington, D.C., National Academies Press.

Cryer, P.E. (2007) Hypoglycemia, functional brain failure, and brain death. *The Journal of clinical investigation*, 117 (4), pp.868–70.

Csapó, J., Martin, T.G., Csapó-Kiss, Z.S. & Házas, Z. (1996) Protein, Fats, Vitamin and Mineral Concentrations in Porcine Colostrum and Milk from Parturition to 60 Days. *International Dairy Journal*, 6 (8), pp.881–902.

Currie, W.B. (1992) *Structure and Function of Domestic Animals*. New York, CRC Press.

Dan, C., Na, Z., Xue-mei, N., Qing-zhang, L. & Xue-jun, G. (2016) Potential Genes for Regulation of Milk Protein Synthesis in Dairy Goat Mammary Gland. *Journal of Northeast Agricultural University (English Edition)*, 23 (1), pp.50–56.

Darwin, Charles, W. & Cord-Ruwisch, R. (2018) Concurrent lactic and volatile fatty acid analysis of microbial fermentation samples by gas chromatography with heat pre-treatment. *Journal of Chromatographic Science*, 56 (1), pp.1–5.

Davis, S.R. Collier, R. J. (1985) Mammary Blood Flow and Regulation of Substrate Supply for Milk Synthesis. *Journal of Dairy Science*, 68(4), pp. 1041–1058.

de Almeida, R.T.R., do Prado, R.M., Porto, C., dos Santos, G.T., Huws, S.A. & Pilau, E.J. (2018) Exploring the rumen fluid metabolome using liquid chromatography-high-resolution mass spectrometry and Molecular Networking. *Scientific Reports*, 8 (1), 1-8.

Deacon, F., Nel, P. J., & Bercovitch, F. B. (2015). Concurrent pregnancy and lactation in wild giraffes (*Giraffa camelopardalis*). *Journal of African Zoology*, 50(4), 331–334.

Denis, B. W. (1917) the influence of the protein intake on the excretion of creatine in man. *J. Biol. Chem*, 30, pp. 47–51.

Derero, A. & Kitaw, G. (2018) Nutritive values of seven high priority indigenous fodder tree species in pastoral and agro-pastoral areas in Eastern Ethiopia. *Agriculture & Food Security*, 7 (1), p.68.

Eaton, A.D., Franson, M.A.H., Clesceri, L.S., Rice, E.W., Greenberg, A.E., American Public Health Association., American Water Works Association. & Water Environment Federation. (2005) *Standard methods for the examination of water and wastewater*. American Public Health Association.

Erfle, J.D., Fisher, L.J. & Sauer, F.D. (1974) Interrelationships Between Blood Metabolites and an Evaluation of Their Use As Criteria of Energy Status of Cows in Early Lactation. *Canadian Journal of Animal Science*, 54 (3), pp.293–303.

Elsden, S.R. (1945) The Fermentation of Carbohydrates in the Rumen of the Sheep. *Journal of Experimental Biology*, 22 (1–2).

Emwas, A.-H.M. (2015) The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research. In: *Methods in molecular biology (Clifton, N.J.)*. pp.161–193.

Enjalbert, F., Nicot, M.C., Bayourthe, C. & Moncoulon, R. (2001) Ketone Bodies in Milk and Blood of Dairy Cows: Relationship between Concentrations and Utilization for Detection of Subclinical Ketosis. *Journal of Dairy Science*, 84 (3), pp.583–589.

Ewer, R.F. (1998) *The Carnivores*. New York, Cornell University Press.

Fantuz, F., Ferraro, S., Todini, L., Piloni, R., Mariani, P. & Salimei, E. (2012) Donkey milk concentration of calcium, phosphorus, potassium, sodium and magnesium. *International Dairy Journal*, 24 (2), pp.143–145.

Farman, M., Tripathi, S. K., Tej, N. K., Nandi, S., Gupta, P. S. P., Mondal, S., & Venkatesh, G. K. (2018). Metabolic Stress Indicators in Ewes (*Ovis aries*) under Post-parturient and High Protein Diet Conditions. *Asian Journal of Animal and Veterinary Advances*, 13(4), 360–368.

Felig P, Owen OE, Wahren J, Cahill GF, Joslin EP (1969) Amino Acid Metabolism during Prolonged Starvation. *The Journal of Clinical investigation* 48: 584–94.

Folch, J.; Lees, M.; Sloane-Stanley, G. H. (1957): A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem*. 226, 497–509.

Fox, P.F. & Mcsweeney, P.L.H. (1998) *Dairy Chemistry and Biochemistry*.

Fox, P.F., Uniacke-Lowe, T., McSweeney, P.L.H. & O'Mahony, J.A. (2015) *Dairy Chemistry and Biochemistry*. Second. New York, Springer.

Frandson, R.D., Wilke, W.L. & Fails, A.D. (2009) *Anatomy and physiology of farm animals*, Wiley-Blackwell, Iowa, JO.

Gastaldi, D., Bertino, E., Monti, G., Baro, C., Fabris, C., Lezo, A., Medana, C., Baiocchi, C., Mussap, M., Galvano, F. & Conti, A. (2010) Donkey's milk detailed lipid composition. *Frontiers in bioscience*, 2 (May 2014), pp.537–546.

Gay, M. C. L., Koleva, P. T., Slupsky, C. M., du Toit, E., Eggesbo, M., Johnson, C. C., Wegienka, G., Shimojo, N., Campbell, D. E., Prescott, S. L., Munblit, D., Geddes, D. T., Kozyrskyj, A. L., Dahl, C., Haynes, A., Hsu, P., Mackay, C., Penders, J., Renz, H., … West, C. (2018). Worldwide variation in human milk metabolome: Indicators of breast physiology and maternal lifestyle. *Journal of Nutrients*, 10(9).

Glasser, F., Doreau, M., Maxin, G. & Baumont, R. (2013) Fat and fatty acid content and composition of forages: A meta-analysis. *Animal Feed Science and Technology*, 185 (1–2), pp.19–34.

Grainger, C., Auldist, M.J., O'Brien, G., Macmillan, K.L. & Culley, C. (2009) Effect of type of diet and energy intake on milk production of Holstein-Friesian cows with extended lactations. *Journal of Dairy Science*, 92 (4), pp.1479–1492.

Grassian, A.R., Parker, S.J., Davidson, S.M., Divakaruni, A.S., Green, C.R., Zhang, X. et al (2014). IDH1 mutations alter citric acid cycle metabolism and increase dependence on oxidative mitochondrial metabolism. *Cancer Res.* 2014; 74 (12), 3317–3331.

Griinari, J.M., Corl, B.A., Lacy, S.H., Chouinard, P.Y., Nurmela, K.V. V. & Bauman, D.E. (2000) Conjugated Linoleic Acid Is Synthesized Endogenously in Lactating Dairy Cows by Δ9-Desaturase. *The Journal of Nutrition*, 130 (9), pp.2285–2291.

Guo, Y.S. & Tao, J.Z. (2018) Metabolomics and pathway analyses to characterize metabolic alterations in pregnant dairy cows on D 17 and D 45 after AI. *Scientific Reports*, 8 (1), p.5973.

Gurmessa, J. & Melaku, A. (2012) Effect of Lactation Stage, Pregnancy, Parity and Age on Yield and Major Components of Raw Milk in Bred Cross Holstein Friesian Cows. *World Journal of Dairy & Food Sciences*, 7 (2), pp.146–149.

Hanuš, O., Samková, E., Křížová, L., Hasoňová, L. & Kala, R. (2018) Role of Fatty Acids in Milk Fat and the Influence of Selected Factors on Their Variability—A Review. *Molecules*, 23 (7), p.1636.

Henao-Velásquez, A.F., Múnera-Bedoya, O.D., Herrera, A.C., Agudelo-Trujillo, J.H., Cerón-Muñoz, M.F., Henao-Velásquez, A.F., Múnera-Bedoya, O.D., Herrera, A.C., Agudelo-Trujillo, J.H. & Cerón-Muñoz, M.F. (2014) Lactose and milk urea nitrogen: fluctuations during lactation in Holstein cows. *Revista Brasileira de Zootecnia*, 43 (9), pp.479–484.

Hettinga, K.A. (2019) Lactose in the dairy production chain. *Lactose*, pp.231–266. Higdon, J. V. & Frei, B. (2006) Coffee and Health: A Review of Recent Human Research. *Critical Reviews in Food Science and Nutrition*, 46 (2), pp.101–123.

Hill, T.M., VandeHaar, M.J., Sordillo, L.M., Catherman, D.R., Bateman, H.G. & Schlotterbeck, R.L. (2011) Fatty acid intake alters growth and immunity in milk-fed calves. *Journal of Dairy Science*, 94 (8), pp.3936–3948.

Holt, C. & Carver, J.A. (2012) Darwinian transformation of a 'scarcely nutritious fluid' into milk. *Journal of Evolutionary Biology*, 25, pp.1253–1263.

Hoppe KE, McKenzie HA. 1974. Comparative studies of alpha-lactalbumin and lysozyme: echidna lysozyme. *Mol. Cell. Biochem*. 3. 93-108.

Hungate, R.E. (1966) *Rumen and Its Microbes.* Elsevier Science.

Iussig, G., Renna, M., Gorlier, A., Lonati, M., Lussiana, C., Battaglini, L.M. & Lombardi, G. (2015) Browsing ratio, species intake, and milk fatty acid composition of goats foraging on alpine open grassland and grazable forestland. *Small Ruminant Research*, 132, pp.12–24.

Iverson, S.J. & Oftedal, O.T. (1995) B. Philogenetic and Ecological Variation in the Fatty Acid Composition of Milks. In: *Handbook of milk composition*. Academic Press Inc.

J. Ellinger, J., A. Chylla, R., L. Ulrich, E., L. Markley, J., Ellinger, J.J., Chylla, R.A., Ulrich, E.L. & Markley, J.L. (2013) Databases and Software for NMR-Based Metabolomics. *Current Metabolomics*, 1 (1), pp.28–40.

Jenkins, T.C. & McGuire, M.A. (2006) Major Advances in Nutrition: Impact on Milk Composition. *J. Dairy Sci.*, 89, p.J. Dairy Sci.

Jeong, J.K.K., Choi, I.S.S., Kang, H.G.G., Hur, T.Y.Y., Jung, Y.H.H. & Kim, I.H.H. (2015) Relationship between serum metabolites, body condition, peri- and postpartum health and resumption of postpartum cyclicity in dairy cows. *Livestock Science*, 181, pp.31–37.

JMP® (1989) JMP® Version 14.

Johnston, M. V., Adams, H.P. & Fatemi, A. (2016) *Neurobiology of Disease*. New York, Oxford University Press.

Jones, A.W. (2002). Reference limits for urine/blood ratios of ethanol in two successive voids from drinking drivers. *J Anal Toxicol*. 26:333–9. 10.1093/jat/26.6.333 [PubMed]

Kaneko, J.J., Harvey, J.W. & Bruss, M.L. (2008) *Clinical Biochemistry of Domestic Animals*. London, Elsevier.

Kapalka, G.M. & Kapalka, G.M. (2010) Substances Involved in Neurotransmission. *Nutritional and Herbal Therapies for Children and Adolescents*, pp.71–99.

Kara, Ç., Kelimeler, A., Propiyonat, K. & Hipokalsemi, K. (2013) Physiological and Metabolic Changes During the Transition Period and the Use of Calcium Propionate for Prevention or Treatment of Hypocalcemia and Ketosis in Periparturient Cows Geçiş Dönemindeki Fizyolojik ve Metabolik Değişimler ile Doğum Sonrası Süt İn. *J. BIOL. ENVIRON. SCI*, 7 (19), pp.9–17.

Kekana, T.W., Nherera-Chokuda, F. V., Muya, M.C., Manyama, K.M. & Lehloenya, K.C. (2018) Milk production and blood metabolites of dairy cattle as influenced by thermal-humidity index. *Tropical Animal Health and Production*, 50 (4), pp.921–924.

Kelly, V., Davis, S., Berry, S., Melis, J., Spelman, R. & Snell, R. (2013) Rapid, quantitative analysis of 3'- and 6'-sialyllactose in milk by flow-injection analysis-mass spectrometry: screening of milks for naturally elevated sialyllactose concentration. *J Dairy Sci 96: 7684–7691*, 96, pp.7684–7691.

Kent, J.C., Arthur, P.G. & Hartmann, P.E. (1998) Citrate, calcium, phosphate and magnesium in sows' milk at initiation of lactation. *The Journal of dairy research*, 65 (1), pp.55–68.

Klein, M.S., Almstetter, M.F., Schlamberger, G., Nürnberger, N., Dettmer, K., Oefner, P.J., Meyer, H.H.D., Wiedemann, S. & Gronwald, W. (2010) Nuclear magnetic resonance and mass spectrometry-based milk metabolomics in dairy cows during early and late lactation. *Journal of Dairy Science, 93, pp.1539–1550*

Kon, S.K. & Cowie, A.T. (2016) *Milk: The Mammary Gland and Its Secretion. Volume 2*. New York, Academic Press Inc.

Konar, A., P.C.T. & Rook., J.A.F. (1971) Concentrations of some water-soluble constituents in milks of cows, sows, ewes and goats. *J. Dairy Res.*, (38), pp.333–341.

Korhonen, M., Vanhatalo, A. & Huhtanen, P. (2002) Effect of Protein Source on Amino Acid Supply, Milk Production, and Metabolism of Plasma Nutrients in Dairy Cows Fed Grass Silage. *Journal of Dairy Science*, 85 (12), pp.3336–3351.

Kumar, Sanjeev, Kumar, B., Kumar, R. & Kumar, Suryamani (2012) Nutritional Features of Goat Milk Review eview. *Indian Journal Dairy Science*, 65(4) (July), pp.266–273.

Lamarre, Simon G., Erica E. Edison, Enoka P. Wijekoon, Margaret E. Brosnan, and John T. Brosnan. 2010. "Suckling Rat Pups Accumulate Creatine Primarily via de Novo Synthesis Rather Than from Dam Milk." *The Journal of Nutrition* 140 (9): 1570–73.

Lämmerhofer, M. & Weckwerth, W. (2013) *Metabolomics in Practise*, Wiley-VCH, Weinheim

Lamp, O., Michael, D., Winfried, O., Manfred, M., Gerd, N. & Björn, K. (2015) Metabolic Heat Stress Adaption in Transition Cows: Differences in Macronutrient Oxidation between Late-Gestating and Early-lactating German Holstein Dairy Cows. Plos One, 10, e0125264.

Lane, J.A., Mariño, K., Naughton, J., Kavanaugh, D., Clyne, M., Carrington, S.D. & Hickey, R.M. (2012) Anti-infective bovine colostrum oligosaccharides: Campylobacter jejuni as a case study. *International Journal of Food Microbiology*, 157 (2), pp.182–188.

Langer, P. (2009) Differences in the composition of colostrum and milk in eutherians reflect differences in immunoglobulin transfer. *Journal of Mammalogy*, 90, pp.332–339.

Lees, H.J., Swann, J.R., Wilson, I.D., Nicholson, J.K. & Holmes, E. (2013) Hippurate: The Natural History of a Mammalian–Microbial Cometabolite. *Journal of Proteome Research*, 12 (4), pp.1527–1546.

Lefèvre CM, Sharp JA, Nicholas KR. 2009. Characterisation of monotreme caseins reveals lineage-specific expansion of an ancestral casein locus in mammals. *Reprod, Fertility Dev*. 21: 1015–1027.

Li, P., Yin, Y.-L., Li, D., Woo Kim, S. & Wu, G. (2007) Amino acids and immune function. *British Journal of Nutrition*, 98 (2), pp.237–252.

Li, Y. & Khanal, S.K. (2017) *Bioenergy : principles and applications*. New Jersey, John Wiley & Sons, Ltd.

Linn, J.G. (1988) Factors Affecting the Composition of Milk from Dairy Cows. In: *Designing Foods: Animal Product Options in the Marketplace*. Washington, DC, The National Academies Press.

Litwack, G. & Litwack, G. (2018) Metabolism of Amino Acids. *Human Biochemistry*, pp.359–394.

Mackle, T.R., Bryant, A.M., Petch, S.F., Hill, J.P. & Auldist, M.J. (1999) *Nutritional Influences on the Composition of Milk from Cows of Different Protein Phenotypes in New Zealand*. *Journal of Dairy Science*, 82, pp.172-180

MacNeil, L. Hill, L., MacDonald, D., Keefe, L., Cormier, J. F., Burke, D. G., & Smith-Palmer, T. (2005) Analysis of creatine, creatinine, creatine-d3 and creatinine-d3 in urine, plasma, and red blood cells by HPLC and GC-MS to follow the fate of ingested creatine-d3. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 827(2), 210–215.

Madende, M., Osthoff, G., Patterton, H. G., Patterton, H. E., Martin, P., & Opperman, D. J. (2015) Characterization of casein and alpha lactalbumin of African elephant (Loxodonta africana) milk. Journal of Dairy Science, 98(12), 8308–8318.

Mahindru, S.N. (2009) *Milk and Milk Products*. New Delhi, APH Publishing.

Mansour, M., Wilhite, D.R. & Rowe, J. (2017) *Guide to ruminant anatomy : dissection and clinical aspects*. John Wiley & Sons, Ltd.

Marczuk, J., Brodzki, P., Brodzki, A. & Korek, L. (2018) Concentration of free amino acids in blood serum of dairy cows with primary ketosis. *Journal of Veterinary Science*, 21 (1), pp.149–156.

Markiewicz-Keszycka, M., Czyzak-Runowska, G., Lipinska, P. & Wójtowski, J. (2013) Fatty acid profile of milk - A review. *Bulletin of the Veterinary Institute in Pulawy*, 57 (2), pp.135–139.

Martini, M., Altomonte, I., Licitra, R. & Salari, F. (2017) Nutritional and Nutraceutical Quality of Donkey Milk. *Journal of Equine Veterinary Science*.

Massart-Leën, A.M. & Massart, D.L. (1981) The use of clustering techniques in the elucidation or confirmation of metabolic pathways. Application to the branched-chain fatty acids present in the milk fat of lactating goats. *The Biochemical journal*, 196 (2), pp.611–8.

Matsuda, H., Asada, T., & Tokumaru, A. M. (2017). Neuroimaging diagnosis for Alzheimer's disease and other dementias. Springer Nature.

Melzer, N., Wittenburg, D., Hartwig, S., Jakubowski, S., Kesting, U., Willmitzer, L., Lisec, J., Reinsch, N. & Repsilber, D. (2013) Investigating associations between milk metabolite profiles and milk traits of Holstein cows. *Journal of Dairy Science*, 96, pp.1521–1534.

Mergenthaler, P., Lindauer, U., Dienel, G.A. & Meisel, A. (2013) Sugar for the brain: the role of glucose in physiological and pathological brain function. *Trends in neurosciences*, 36 (10), pp.587–97.

M'hamed, D., Faverdin, P. & Verité, R. (2001) Effects of the level and source of dietary protein on intake and milk yield in dairy cows. *Animal Research*, 50 (3), pp.205–211.

Miller, W.J. (William J. (1979) *Dairy cattle feeding and nutrition*. Academic Press.

Mitchell, G. & Skinner, J.D. (2010) Giraffe Thermoregulation: a review. *Transactions of the Royal Society of South Africa*, 59 (2), pp.109–118.

Mizrahi, I. (2013) Rumen symbioses. In: The Prokaryotes: *Prokaryotic Biology and Symbiotic Associations*. Springer-Verlag Berlin Heidelberg, pp.533–544.

Moran, J. (2005) *Tropical Dairy Farming: Feeding Management for Small Holder Dairy Farmers in the Humid Tropics*. Oxford, Department of Primary Industries.

MosaviI, G.R., Fatahnia, F., Mirzaei Alamouti, H.R., Mehrabi, A.. & KohiII, D.H. (2012) Effect of dietary starch source on milk production and composition of lactating Holstein cows. *South African Journal of Animal Science*, 42 (3), pp.201–209.

Murgia, A., Scano, P., Contu, M., Ibba, I., Altea, M., Bussu, M., Demuru, M., Porcu, A. & Caboni, P. (2016) Characterization of donkey milk and metabolite profile comparison with human milk and formula milk. *LWT - Food Science and Technology y*, 74, pp.427–433.

Najmus Saqib, M., Subhan Qureshi, M., Ullah Khan, R., Saqib, M.N., Qureshi, M.S., Khan, R.U., Najmus, M.N., Subhan, M.S. & Ullah, R.U. (2018) Changes in postpartum metabolites and resumption of ovarian cyclicity in primiparous and multiparous dairy cows. *Applied Biological Chemistry*, 61 (1), pp.107–111.

Nakamura, T., Urashima, T., Mizukami, T., Fukushima, M., Arai, I. & Senshu, T. (2003) Composition and oligosaccharides of a milk sample of the giant panda, Ailuropoda melanoleuca. *Comparative Biochem- istry and Physiology B-Biochemistry & Molecular Biology*, 135, pp.439–448.

Nantapo, C.T.W., Muchenje, V. & Hugo, A. (2014) Atherogenicity index and health-related fatty acids in different stages of lactation from Friesian, Jersey and Friesian × Jersey cross cow milk under a pasture-based dairy system. *Food Chemistry*, 146, pp.127–133.

Neville, M.C., Keller, R.P. & Allen, J.C. (1994) Calcium partitioning in human and bovine milk. *Journal of Dairy Science*, 77, pp.1964–1975.

Nielsen, J. & Jewett, M.C. (2007) *Metabolomics*. Berlin, Heidelberg, Springer Berlin Heidelberg.

O'Callaghan, T., Vázquez-Fresno, R., Serra-Cayuela, A., Dong, E., Mandal, R., Hennessy, D., McAuliffe, S., Dillon, P., Wishart, D., Stanton, C. & Ross, R. (2018) Pasture Feeding Changes the Bovine Rumen and Milk Metabolome. *Metabolites*, 8 (2), p.27.

Oftedal, O.T., Eisert, T. & Barrell, G.K. (1995) Comparitive Analysis of Nonhuman Milks. In: R. G. Jensen ed. *Handbook of milk composition.* Academic Press Inc., pp.749–768.

Oftedal, O.T. (2012) The evolution of milk secretion and its ancient origins. *Animal Science Journal*, 6, pp.355–368.

Oftedal, O. T., Boness, D. T. & Tedman, R. A. 1987 The behaviour, physiology, and anatomy of lactation in the pinnipedia. Curr. Mammal. 1, 175–245.

Oftedal, O., Eisert, R. & Barrell, G. (2014) Comparison of analytical and predictive methods for water, protein, fat, sugar, and gross energy in marine mammal milk. , 97, pp.4713–4732.

Ogola, H., Shitandi, A. & Nanua, J. (2007) Effect of mastitis on raw milk compositional quality. *Journal of Veterinary Science*, 8 (3), pp.237–242.

Orskov, E.R. (1986) Starch Digestion and Utilization in Ruminants. *Journal of Animal Science*, 63 (5), pp.1624–1633.

Osthoff, G., De Waal, H.O., Hugo, A., De Wit, M. & Botes, P. (2005) Milk composition of a free-ranging African elephant (Loxodonta africana) cow during early lactation. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, 141 (2), pp.223–229.

Osthoff, G., Dickens, L., Urashima, T., Bonnet, S.L., Uemura, Y. & van der Westhuizen, J.H. (2008) Structural characterization of oligosaccharides in the milk of an African elephant (Loxodonta africana africana). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 150 (1), pp.74–84.

Osthoff, G., Hugo, A. & de Wit, M. (2009) Comparison of the milk composition of free-ranging blesbok, black wildebeest and blue wildebeest of the subfamily Alcelaphinae (family: Bovidae). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, 154 (1), pp.48–54.

Osthoff, G., Hugo, A. & De Wit, M. (2006) The composition of cheetah (Acinonyx jubatus) milk. *Comparative Biochemistry and Physiology*, 145, pp.265–269.

Osthoff, G., Hugo, A., Madende, M., Deacon, F. & Nel, P.J. (2017) Milk composition of free-ranging red hartebeest, giraffe, Southern reedbuck and warthog and a phylogenetic comparison of the milk of African Artiodactyla. *Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology*, 204, pp.93–103.

Osthoff, G. (2016) Milk: Milks of Non-dairy Mammals. In: *Reference Module in Food Science*. Elsevier.

Osthoff, G., Hugo, A., De Wit, M. & Nguyen, T.P.M. (2009) The chemical composition of milk from free-ranging African buffalo (Syncerus caffer). *South African Journal of Wildlife Research*, 39 (April), pp.97–102.

Owen, F.G., Kubik, D.J. & Larson, L.L. (1984) *Factors Affecting Milk Protein and Related Recommendations for Dairymen*.

Palmquist, D. (2006) Milk Fat: Origin of Fatty Acids and Influence of Nutritional Factors Thereon. In: P.Fox & P. L. McSweeney eds. *Advanced Dairy chemistry Volume 2: Lipids*. New York, Springer Science Business Media.

Park, Y.W. & Haenlein, G.F.W. (2013) *Milk and Dairy Products in Human Nutrition : Composition, Production and Health.* Wiley.

Park, Y.Q. (2016) Goat Milk : Composition, Characteristics. *Agricultural Research Station, Fort Valley State University, Fort Valley, Georgia, U.S.A.*

Parker, B.K. (2005) *Giraffes*. Lerner Publications Company, Minnesota.

Perry, T.W. (1980) *Beef cattle feeding and nutrition*. London, Academic Press.

Pohn, G., Albert, C., Csapó-Kiss, Z. & Csapó, J. (2009) *Influence of mastitis on D-amino acid content of milk. Alimentaria, (2), pp.31-43.*

Pontarotti, P. (2014) *Evolutionary Biology: Genome Evolution, Speciation, Coevolution and Origin of Life*. New York, Springer.

Potter, J.S. & Clauss, M. (2005) Mortality of Captive Giraffe (Giraffa Camelopardalis) Associated With Serous Fat Atrophy: a Review of Five Cases At Auckland Zoo. *Journal of Zoo and Wildlife Medicine*, 36 (2), pp.301–307.

Prager EM, Wilson AC. 1988. Ancient origin of lactalbumin from lysozyme: analysis of DNA and amino acid sequences. *J. Mol. Evol*. 27: 326-335.

Probert, M. E., P. S. Carberry, R. L. McCown, and J. E. Turpin, 'The Concentration of Creatine and Creatine Phosphate in Sow Colostrum and Milk throughout Lactation and Weaning', *Australian Journal of Agricultural Research*, 48 (1997), 1105–10.

Psychogios, N., Hau, D.D., Peng, J., Guo, A.C., Mandal, R., Bouatra, S., Sinelnikov, I., Krishnamurthy, R., Eisner, R., Gautam, B., Young, N., Xia, J., Knox, C., Dong, E., Huang, P., Hollander, Z., Pedersen, T.L., Smith, S.R., Bamforth, F., Greiner, R., McManus, B., Newman, J.W., Goodfriend, T. & Wishart, D.S. (2011) The Human Serum Metabolome D. Flower ed. *PLoS ONE*, 6 (2), pp.1-23

Qasba PK, Kumar S. 1997. Molecular divergence of lysozyme and α-lactalbumin. *Crit. Rev. Biochem. Mol. Biol*. 32. 255-306.

Randhawa, S.N.S., Ranjan, R., Singh, R. & Chand, N. (2014) Diagnosis and Management of Negative Energy Balance and Associated Production Diseases in Bovines. *Intas Polivet*, 15 (2), pp.497–503.

Rego, O.A., Cabrita, A.R.J., Rosa, H.J.D., Alves, S.P., Duarte, V., Fonseca, A.J.M., Vouzela, C.F.M., Pires, F.R. & Bessa, R.J.B. (2016) Changes in milk production and milk fatty acid composition of

cows switched from pasture to a total mixed ration diet and back to pasture. *Italian Journal of Animal Science*, 15 (1), pp.76–86.

Reich, C.M. & Arnould, J.P.Y. (2007) Evolution of Pinnipedia lactation strategies: A potential role for α-lactalbumin? *Biology Letters*, 3 (5), pp.546–549.

Rijnkels M. 2002. Multispecies comparison of the casein gene loci and evolution of casein gene family. *J. Mammary Gland Biol. Neoplasia* 7: 327-345.

Rius, A.G., Appuhamy, J.A.D.R.N., Cyriac, J., Kirovski, D., Becvar, O., Escobar, J., McGilliard, M.L., Bequette, B.J., Akers, R.M. & Hanigan, M.D. (2010) Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. *Journal of Dairy Science*, 93 (7), pp.3114–3127.

Röder, P. V, Wu, B., Liu, Y. & Han, W. (2016) Pancreatic regulation of glucose homeostasis. *Experimental & molecular medicine*, 48 (3), pp.1-19.

Rohlfs, E. M., Garner, S. C., Mar, M.H. & Zeisel, S. H. (1993). Glycerophosphocholine and phosphocholine are the major choline metabolites in rat milk. The Journal of Nutrition, 123(10), 1762–1768.

Ruska, D. & Jonkus, D. (2015) Crude Protein and Non-protein Nitrogen Content in Dairy Cow Milk. *Proceedings of the Latvia University of Agriculture*, 32 (1), pp.36–40.

Sauer, J.J.C., Skinner, J.D. & Neitz, A.W.H. (1982) Seasonal utilization of leaves by giraffes (*Giraffa camelopardalis)*, and the relationship of the seasonal utilization to the chemical composition of the leaves. *South African Journal of Zoology*, 17 (4), pp.210–219.

Scano, P., Murgia, A., Pirisi, F.M. & Caboni, P. (2014) A gas chromatography-mass spectrometry-based metabolomic approach for the characterization of goat milk compared with cow milk. *Journal of Dairy Science*, 97, pp.6057–6066.

Schingoethe, D.J. (1996) Dietary influence on protein level in milk and milk yield in dairy cows. *Animal Feed Science and Technology*, 60 (3–4), pp.181–190.

Schwab, C. G., Satter, L. D. and Clay, A. B. (1976). Response of lactating cows to abomasal infusions of amino acids. J. Dairy Sci. 59:1254. [PubMed]

Shin, E.-K., Jeong, J.-K., Choi, I.-S., Kang, H.-G., Hur, T.-Y., Jung, Y.-H. & Kim, I.-H. (2015) Relationships among ketosis, serum metabolites, body condition, and reproductive outcomes in dairy cows. *Theriogenology*, 84 (2), pp.252–260.

Shorrocks, B. (2016) *The Giraffe: Biology, Ecology, Evolution and Behaviour*. Chichester, UK, John Wiley & Sons, Ltd.

Skibiel, A.L., Downing, L.M., Orr, T.J. & Hood, W.R. (2013) The evolution of the nutrient composition of mammalian milks A. Russell ed. *Journal of Animal Ecology*, 82 (6), pp.1254–1264.

Smilowitz, J.T., O'Sullivan, A., Barile, D., German, J.B., Lönnerdal, B. & Slupsky, C.M. (2013) The human milk metabolome reveals diverse oligosaccharide profiles. *The Journal of nutrition*, 143 (11), pp.1709–18.

Solaiman, S.G. (2010) *Goat Science and Production*. John Wiley and Sons, Inc., Hoboken.

Sparkman, O.D., Penton, Z.E., Kitson, F.G., Sparkman, O.D., Penton, Z.E. & Kitson, F.G. (2011) Amino Acids. *Gas Chromatography and Mass Spectrometry*, pp.265–271.

Sun, H.-Z., Shi, K., Wu, X.-H., Xue, M.-Y., Wei, Z.-H., Liu, J.-X. & Liu, H.-Y. (2017) Lactation-related metabolic mechanism investigated based on mammary gland metabolomics and 4 biofluids' metabolomics relationships in dairy cows. *BMC Genomics*, 18 (936)

Sundrum, A. (2015) Metabolic Disorders in the Transition Period Indicate that the Dairy Cows' Ability to Adapt is Overstressed. *Animals: an open access journal from MDPI*, 5 (4), pp.978–1020.

Sutton, J.D., Dhanoa, M.S., Morant, S. V., France, J., Napper, D.J. & Schuller, E. (2003) Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. *Journal of Dairy Science*, 86 (11), pp.3620–3633.

Tallima, H. & El Ridi, R. (2018) Arachidonic acid: Physiological roles and potential health benefits - A review. *Journal of advanced research*, 11, pp.33–41.

ten Bruggencate, S.J., Bovee-Oudenhoven, I.M., Feitsma, A.L., van Hoffen, E. & Schoterman, M.H. (2014) Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. *Nutrition Reviews*, 72 (6), pp.377–389.

Thung, S.F. & Norwitz, E.R. (2009) Endocrine Diseases of Pregnancy. In: *Yen & Jaffe's Reproductive Endocrinology*. Elsevier Inc., pp.615–658.

Tordiffe, A.S.W., Wachter, B., Heinrich, S.K., Reyers, F. & Mienie, L.J. (2016) Comparative Serum Fatty Acid Profiles of Captive and Free-Ranging Cheetahs (Acinonyx jubatus) in Namibia G. Umapathy ed. *PLOS ONE*, 11 (12), p.e0167608.

Trabado, S., Al-Salameh, A., Croixmarie, V., Masson, P., Corruble, E., Fève, B., Colle, R., Ripoll, L., Walther, B., Boursier-Neyret, C., Werner, E., Becquemont, L., & Chanson, P. (2001) The human plasma-metabolome: Reference values in 800 French healthy volunteers; Impact of cholesterol, gender and age. PLoS ONE, 12(3).

Tyasi, T., Gxasheka, M. & Tlabela, C. (2015) Assessing the effect of nutrition on milk composition of dairy cows: A review. *International Journal of Current Science*, 2015 (September), pp.56–63.

Urashima, T., Taufik, E., Fukuda, K. & Asakuma, S. (2013) Recent Advances in studies on milk oligosaccharides of cows and other domestic farm animals. *Bioscience Biotechnology and Biochemistry*, 77 (49), pp.455– 466.

Valdes, E. V. & Schlegel, M. (2012) Advances in Giraffe Nutrition. *Fowler's Zoo and Wild Animal Medicine*, pp.612–618.

Vance, D.. & Vance, J.. (2008) *Biochemistry of Lipids, Lipoproteins and Membranes*. First Edition. Amsterdam, Elsevier B. V.

Verma, K.S., Pandey, R. & Ayachi, A. (2014) Nutritional Assessment of different parts of Acacia catechu collected from Central India. *International Journal of Pharmaceutical Sciences and Research*, 5 (7), pp.2980–2986.

Walstra, P. (1999) *Dairy Technology*. New York, CRC Press.

Walstra, P., Wouters, J.T.. & Geurts, T.J. (2005) *Dairy Science and Technology, Second Edition*. New York, CRC PressTalylor & Francis Group.

Webster, J. (2019) *Calf Husbandry, Health And Welfare.* New York, CRC Press.

Weiss, G.A. & Hennet, T. (2012) The Role of Milk Sialyllactose in Intestinal Bacterial Colonization. *Advances in Nutrition*, 3 (3), pp.483S-488S.

Winek, C.L. & Esposito, F.M. (1985) Blood alcohol concentrations: factors affecting predictions. *Legal medicine*, pp.34–61.

Wishart, D.S., Knox, C., Guo, A.C., Eisner, R., Young, N., Gautam, B., Hau, D.D., Psychogios, N., Dong, E., Bouatra, S., Mandal, R., Sinelnikov, I., Xia, J., Jia, L., Cruz, J.A., Lim, E., Sobsey, C.A., Shrivastava, S., Huang, P., Liu, P., Fang, L., Peng, J., Fradette, R., Cheng, D., Tzur, D., Clements, M., Lewis, A., De Souza, A., Zuniga, A., Dawe, M., Xiong, Y., Clive, D., Greiner, R., Nazyrova, A., Shaykhutdinov, R., Li, L., Vogel, H.J. & Forsythe, I. (2009) HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Research*, 37 (Database), pp.D603–D610.

Wishart, D.S., Tzur, D., Knox, C., Eisner, R., Guo, A.C., Young, N., Cheng, D., Jewell, K., Arndt, D., Sawhney, S., Fung, C., Nikolai, L., Lewis, M., Coutouly, M.-A., Forsythe, I., Tang, P., Shrivastava, S., Jeroncic, K., Stothard, P., Amegbey, G., Block, D., Hau, D.D., Wagner, J., Miniaci, J., Clements, M., Gebremedhin, M., Guo, N., Zhang, Y., Duggan, G.E., MacInnis, G.D., Weljie, A.M., Dowlatabadi, R., Bamforth, F., Clive, D., Greiner, R., Li, L., Marrie, T., Sykes, B.D., Vogel, H.J. & Querengesser, L. (2007) HMDB: the Human Metabolome Database. *Nucleic Acids Research*, 35 (Database), pp.D521–D526.

Wishart, D.S., Jewison, T., Guo, A.C., Wilson, M., Knox, C., Liu, Y., Djoumbou, Y., Mandal, R., Aziat, F., Dong, E., Bouatra, S., Sinelnikov, I., Arndt, D., Xia, J., Liu, P., Yallou, F., Bjorndahl, T., Perez-Pineiro, R., Eisner, R., Allen, F., Neveu, V., Greiner, R. & Scalbert, A. (2012) HMDB 3.0—The Human Metabolome Database in 2013. *Nucleic Acids Research*, 41 (D1), pp.D801–D807.

Wishart, D.S., Feunang, Y.D., Marcu, A., Guo, A.C., Liang, K., Vázquez-Fresno, R., Sajed, T., Johnson, D., Li, C., Karu, N., Sayeeda, Z., Lo, E., Assempour, N., Berjanskii, M., Singhal, S., Arndt, D., Liang, Y., Badran, H., Grant, J., Serra-Cayuela, A., Liu, Y., Mandal, R., Neveu, V., Pon, A., Knox, C., Wilson, M., Manach, C. & Scalbert, A. (2018) HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Research*, 46 (D1), pp.D608–D617.

Wong, N. P. (1988). Fundamentals of Dairy Chemistry. Van Nostrand Reinhold Company Inc.

Woods, V.B. & Fearon, A.M. (2009) Dietary sources of unsaturated fatty acids for animals and their transfer into meat, milk and eggs: A review. *Livestock Science*, 126 (1–3), pp.1–20.

www.weathersa.co.za (2019) South African Weather Service.

Xia, J., Broadhurst, D.I., Wilson, M. & Wishart, D.S. (2013) Translational biomarker discovery in clinical metabolomics: An introductory tutorial. *Metabolomics*, 9 (2), pp.280–299.

Xia, J., Mandal, R., Sinelnikov, I. V., Broadhurst, D. & Wishart, D.S. (2012) MetaboAnalyst 2.0-a comprehensive server for metabolomic data analysis. *Nucleic Acids Research*, 40 (W1).

Xia, J., Psychogios, N., Young, N. & Wishart, D.S. (2009) MetaboAnalyst: A web server for metabolomic data analysis and interpretation. *Nucleic Acids Research*, 37 (SUPPL. 2).

Xia, J., Sinelnikov, I. V., Han, B. & Wishart, D.S. (2015) MetaboAnalyst 3.0-making metabolomics more meaningful. *Nucleic Acids Research*, 43 (W1), pp.W251–W257.

Xia, J., Sinelnikov, I. V. & Wishart, D.S. (2011) MetATT: A web-based metabolomics tool for analyzing time-series and two-factor datasets. *Bioinformatics*, 27 (17), pp.2455–2456.

Xia, J. & Wishart, D.S. (2011) Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nature Protocols*, 6 (6), pp.743–760.

Xia, J. & Wishart, D.S. (2016) Using metaboanalyst 3.0 for comprehensive metabolomics data analysis. *Current Protocols in Bioinformatics*, 2016, pp.14.10.1-14.10.91.

Xia, J. & Wishart, D.S. (2010) MSEA: A web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. *Nucleic Acids Research*, 38 (SUPPL. 2).

Xia, J. & Wishart, D.S. (2011) Metabolomic data processing, analysis, and interpretation using MetaboAnalyst. *Current Protocols in Bioinformatics*, (SUPPL. 34), pp.1–48.

Xia, J., Wishart, D.S. & Valencia, A. (2011) MetPA: A web-based metabolomics tool for pathway analysis and visualization. In: *Bioinformatics*. Oxford University Press, pp.2342–2344.

Xuanzhen, L., Mingxi, L., Jianqiu, Y., Zhihe, Z., Xiangming, H., Jingchao, L., Zhi, Y., Liu, X.Z., Li, M.X., Yu, J.Q., Zhang, Z.H., Huang, X.M., Lan, J.C., Xuanzhen, L., Mingxi, L., Jianqiu, Y., Zhihe, Z., Xiangming, H., Jingchao, L. & Zhi, Y. (2005) Composition of captive giant panda milk. *Zoo Biology*, 24 (41), pp.393–398.

Yan, J., Ding, J. & Liang, X. (2017) Chromatographic methods for the analysis of oligosaccharides in human milk. *Analytical Methods*, 9 (7), pp.1071–1077.

Yost, W.M., Young, J.W., Schmidt, S.P. & McGilliard, A.D. (1977) Gluconeogenesis in Ruminants: Propionic Acid Production from a High-Grain Diet Fed to Cattle. *The Journal of Nutrition*, 107 (11), pp.2036–2043.

Zadow, J.G. (1992) *Whey and Lactose Processing*. New York, Elsevier Science Publishers LTD.

Zhang, T., Zhang, R., Zhang, L., Zhang, Z., Hou, R. & Wang, H. (2015) Changes in the Milk Metabolome of the Giant Panda (Ailuropoda melanoleuca) with Time after Birth – Three Phases in Early Lactation and Progressive Individual Differences, pp.1–19.

Zhang, Z., Hou, R., Lan, J., Wang, H., Kurokawa, H., Takatsu, Z., Kobayashi, T., Koie, H., Kamata, H., Kanayama, K. & Watanabe, T. (2016) Analysis of the breast milk of giant pandas (Ailuropoda melanoleuca) and the preparation of substitutes. *The Journal of veterinary medical science*, 78 (5), pp.747–54.

Zivkovic, A.M. & Barile, D. (2011) Bovine Milk as a Source of Functional Oligosaccharides for Improving Human Health. *Advances in Nutrition*, 2 (3), pp.284–289.

Chapter 7 - Appendix A

Glycolysis, Krebs cycle, gluconeogenesis, pentose phosphate pathway, and energy metabolism related metabolites

Table A 1: Detected metabolites associated with glycolysis, the krebs cycle, gluconeogenesis, pentose phosphate pathway, and energy metabolism in the milk of mammals representative from different taxonomic orders. The square symbol indicates detected metabolites and the negative symbol indicates undetected metabolites.

Metabolite	Brown Swiss & Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Method of analysis	NMR, GC-MS	GC-MS	GC-MS	HILIC–HRMS	GC-MS	NMR
1,3-Dihydroxyacetone	-	■	-	-	-	-
2-oxoglutarate	-	-	-	-	-	■
3-Phosphoglycerate	-	-	■	-	-	-
Aconitate	-	-	■	-	-	■
Citrate	■	-	■	■	■	■
Creatine	-	-	-	■	-	■
Creatinine	■	-	-	-	-	■
Fructose-6-phosphate	-	■	■	-	-	-
Fumarate	■	-	-	-	-	■
Glycerol	-	■	-	-	-	-
Itaconate	-	■	■	-	-	-
Lactate	■	■	■	-	■	■
Malate	■	-	■	-	■	-
Mannose-6-phosphate	-	-	■	-	-	-
Phosphocreatine	■	-	-	■	-	■
Phosphocreatinine	■	-	-	-	-	-
Phosphoenolpyruvate	-	■	-	-	-	-
Ribulose-5-phosphate	-	■	-	-	-	-
Pyruvate	■	■	-	-	-	■
Succinate	■	-	■	■	■	■

Metabolite	Brown Swiss & Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
[1] (Klein et al., 2010); [2] (Melzer et al., 2013); [3] (Caboni et al., 2016); [4] (Zhang et al., 2015); [5] (Murgia et al. 2016); [6] (Smilowitz et al. 2013)						

Amino acid metabolism, nucleic acid metabolism and nitrogen balance related metabolites

Table A 2: Metabolites relevant to amino acid metabolism, nucleic acid related metabolism, and nitrogen balance in the milk of mammals representative from different taxonomic orders. The square symbol indicates detected metabolites and the negative symbol indicates undetected metabolites.

Metabolite	Brown Swiss & Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Method of analysis	NMR, GC-MS	GC-MS	GC-MS	HILIC–HRMS	GC-MS	NMR
2-amino adipic acid	-	■	-	-	-	-
2-aminobutyrate	-	-	-	-	-	■
2-Piperidinecarboxylic acid	-	■	-	-	-	-
3-Methylhistidine	■	-	-	-	-	-
5-Guanidino-2-oxopentanoate	-	-	-	■	-	-
Acetylcarnitine	-	-	-	-	-	■
Alanine	■	■	■	■	■	■
Arginine	-	■	-	■	-	■
Asparagine	-	■	-	-	-	■
Aspartate	■	■	-	■	■	■
Betaine	■	-	-	■	-	■
Carnitine	■	-	-	-	-	■
Cysteine	-	-	-	-	-	■
Cytidine	-	-	-	-	-	■
Glutamate	■	-	-	■	■	■
Glutamine	-	-	-	-	-	■
Glycine	■	■	■	-	-	-
Histidine	-	-	-	■	-	■
Hypoxanthine	-	-	-	-	-	■

Metabolite	Brown Swiss & Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Isoleucine	■	-	■	■	■	■
Kynurenine	-	■	-	-	-	-
Leucine	■	■	-	■	-	■
Lysine	■	-	-	■	-	■
Methionine	■	■	-	■	-	■
N-Acetylputrescine	-	-	-	■	-	-
Ornithine	■	-	-	-	-	-
Orotic acid	-	■	-	-	■	■
Phenylalanine	■	■	-	■	■	■
Phosphoethanolamine	-	■	-	-	-	-
Proline	■	-	■	■	-	■
Pyroglutamic acid	-	■	■	-	■	■
Serine	-	-	■	■	■	■
Spermidine	-	■	-	-	-	-
Taurine	■	-	-	■	-	■
Threonine	■	-	-	■	■	■
Tryptophan	■	■	-	■	-	■
Tyrosine	■	■	-	■	■	■
Uracil	-	■	■	-	■	-
Urea	-	■	■	■	■	■
Uridine	-	-	-	-	-	■
Valine	■	-	■	■	■	■
α-Aminoadipic acid	■	-	-	-	-	-
α-Aminobutyric acid	■	-	-	-	-	-
β-Citryl-L-glutamate	-	-	-	■	-	-

[1] (Klein et al. 2010); [2] (Melzer et al. 2013) [3] (Caboni et al. 2016); [4] (Zhang et al. 2015; Zhang et al. 2016); [5] (Murgia et al. 2016); [6] (Smilowitz et al. 2013)

Saccharides, sugar alcohols, and related metabolites

Table A 3: Metabolites relevant to saccharides, sugar alcohols, and related molecules in the milk of mammals representative from different taxonomic orders. The square symbol indicates detected metabolites and the negative symbol indicates undetected metabolites.

Metabolite	Brown Swiss & Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Method of analysis	NMR, GC-MS	GC-MS	GC-MS	HILIC–HRMS	GC-MS	NMR
1,3-dihydroxyacetone	-	■	-	-	-	-
2-Deoxyribose	-	-	■	-	-	-
2'-Fucosyllactose	-	-	-	-	-	■
3'-Fucosyllactose	-	-	-	■	-	■
3'-Sialyllactose	-	-	-	■	-	■
6'-Sialyllactose	-	-	-	■	-	■
Altrose	-	-	■	-	-	-
Arabitol	-	■	■	-	-	-
Cellubiose	-	-	■	-	-	-
Citramalate	-	-	-	■	-	-
Fructose	-	-	■	■	■	-
Fucose	-	-	■	-	■	■
Fucosylisoglobotriose	-	-	-	■	-	-
Galactitol	-	■	-	-	-	-
Galactose	■	-	■	-	■	■
Glucaric acid-1,4-lactone	-	■	-	-	-	-
Gluconic acid	-	■	-	-	-	-
Gluconic acid-6-phosphate	-	■	-	-	-	-
Glucopyranoside	-	■	-	-	-	-
Glucosamine	-	■	-	-	-	-
Glucose	■	-	■	-	■	■
Inositol	-	-	■	-	-	-
Isoglobotriose	-	-	-	■	-	-

Metabolite	Brown Swiss & Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Lacto- N-neotetraose	-	-	-	-	-	■
Lactodifucotetraose	-	-	-	-	-	■
Lacto-N-fucopentaose	-	-	-	-	-	■
Lacto-N-tetraose	-	-	-	-	-	■
Lactose	■	■	■	■	■	■
Levoglucosan	-	-	■	-	-	-
Maltose	-	-	■	-	-	-
Mannitol	-	-	■	-	■	-
Methanol	-	-	-	-	-	■
Myo-inositol	-	-	■	-	■	■
Myoinositol-1-phospahte	-	■	-	-	-	-
N-acetyl Galactosamine	-	■	-	-	-	-
Palatinose	-	-	■	-	■	■
Ribose	-	-	■	-	-	-
Scyllo-inositol	-	-	■	-	■	■
Sedoheptulose	-	■	-	-	-	-
Sorbitol	-	-	■	■	-	-
Talose	-	-	-	-	■	-

[1] (Klein et al. 2010); [2] (Melzer et al. 2013) [3] (Caboni et al. 2016;); [4] (Zhang et al. 2015; Zhang et al. 2016); [5] (Murgia et al. 2016); [6] (Smilowitz et al. 2013)

Organic acids and related metabolites

Table A 4: Metabolites associated with organic acids and related molecules in the milk of mammals representative from different taxonomic orders. The square symbol indicates detected metabolites and the negative symbol indicates undetected metabolites.

Metabolite	Brown Swiss and Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Method of analysis	NMR, GC-MS	GC-MS	GC-MS	HILIC–HRMS	GC-MS	NMR
Acetate	■	-	-	-	-	■
Ascorbate	-	-	-	-	-	■

Metabolite	Brown Swiss and Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Azelaic acid	-	-	-	-	-	■
Benzoic acid	-	■	-	-	-	-
Cinnamic acid	-	■	-	-	-	-
Hippuric	-	-	-	-	-	■
Methyl maleic acid	-	-	■	-	-	-

[1] (Klein et al. 2010); [2] (Melzer et al. 2013) [3] (Caboni et al. 2016); [4] (Zhang et al. 2015; Zhang et al. 2016); [5] (Murgia et al. 2016); [6] (Smilowitz et al. 2013)

Fatty acids and related metabolites

Table A 5: Metabolites associated with fatty acids and fatty acid derivatives in the milk of mammals representative from different taxonomic orders. The square symbol indicates detected metabolites and the negative symbol indicates undetected metabolites. Glycerol-2-phosphate is the most commonly occurring metabolite.

Metabolite	Brown Swiss and Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Method of analysis	NMR, GC-MS	GC-MS	GC-MS	HILIC–HRMS	GC-MS	NMR
2-oxooctadecanoic acid	-	-	-	■	-	-
4-trimethylammoniobutanoate	-	-	-	■	-	-
Butyrate	-	■	-	-	-	■
Caprate	-	-	-	-	-	■
Eicosenoic acid	-	-	-	■	-	-
Formate	-	-	-	-	-	■
Glycero-3-phosphocholine	-	-	-	■	-	■
glycero-3-phosphoethanolamine	-	-	-	■	-	-
Glycerol	-	■	-	-	-	-
Glycerol-2-phosphate	■	■	-	-	-	■
Glycerol-3-phosphate	-	■	-	-	-	-
Glycerophosphocholine	■	-	-	-	-	-
Linoleic acid	-	-	-	■	-	-

Palmitic acid	-	-	-	-	■	-
Tetradecenoic acid	-	-	-	■	-	-
β-hydroxybutyrate (3HBA)	■	-	-	-	-	-

[1] (Klein et al. 2010); [2] (Melzer et al. 2013) [3] (Caboni et al. 2016); [4] (Zhang et al. 2015; Zhang et al. 2016); [5] (Murgia et al. 2016); [6] (Smilowitz et al. 2013)

Vitamin related metabolites

Table A 6: Metabolites associated with vitamins in the milk of mammals representative from different taxonomic orders. The square symbol indicates detected metabolites and the negative symbol indicates undetected metabolites.

Metabolite	Brown Swiss and Simmental cows[1]	Holstein cow[2]	Goat[3]	Giant panda[4]	Donkey[5]	Human[6]
Method of analysis	NMR, GC-MS	GC-MS	GC-MS	HILIC–HRMS	GC-MS	NMR
4-pyridoxate	-	-	-	■	-	-
8-Amino-7-Oxononanoate	-	-	-	■	-	-
Choline	■	-	-	-	-	■
Niacinamide	-	-	-	-	-	■
Pantothenate	-	-	-	-	-	■
Phosphocholine	■	■	-	■	-	■
Thiazole	-	■	-	-	-	-

[1] (Klein et al. 2010); [2] (Melzer et al. 2013) [3] (Caboni et al. 2016); [4] (Zhang et al. 2015; Zhang et al. 2016); [5] (Murgia et al., 2016); [6] (Smilowitz et al. 2013)

Chapter 8 - Appendix B

Table B 1: Giraffe sample information

Sample	Reserve[1]	Milk collected (ml)	Pregnancy status	Lactation time	Blood collected
F1	Rooiport	14	N	3.5 months	Y
BF1	Rooiport	NS	N	-	Y
F2	Rooiport	11	N	3.5 months	Y
F3	Rooiport	6	Y	4.5 months	Y
F4	Rooiport	-	Y	-	N
F5	Rooiport	-	Y	-	Y
F6	Rooiport	17	N	4.5 months	Y
F7	Rooiport	-	Y (14 - 15 months)	-	N
BF7	Rooiport	2	Y	-	Y
F8	Rooiport	-	Y (10 - 12 months)	-	Y
F9	Rooiport	11	Y (9 - 11 months)	-	Y
F10	Rooiport	-	Y (9 - 11 months)	-	Y
F11	Rooiport	15	N	2.5 months	Y
SVF1	Sandveld	NS	N	-	Y
SVF2	Sandveld	-	Y	-	Y
M1	Rooiport	-	-	-	Y
M2	Rooiport	-	-	-	N
M3	Rooiport	-	-	-	N
M4	Rooiport	-	-	-	N
M5	Rooiport	-	-	-	Y
M6	Rooiport	-	-	-	Y
M7	Rooiport	-	-	-	N

Sample	Reserve[1]	Milk collected (ml)	Pregnancy status	Lactation time	Blood collected
BM1	Rooiport	-	-	-	N
BM2	Rooiport	-	-	-	Y
BM3	Rooiport	-	-	-	Y
BM4	Rooiport	-	-	-	Y
BM5	Rooiport	-	-	-	Y
BM6	Rooiport	-	-	-	N
BM7	Rooiport	-	-	-	Y
Mxam	Rooiport	-	-	-	Y
Mxred	Rooiport	-	-	-	Y

Table B 2: p-values based on sex and year of serum metabolites in male and female giraffes. p values based on year include only the giraffes located in Rooiport game reserve.

Sex		Year	
Metabolite	p-value	Metabolite	p-value
Creatinine	0.0082	Citric acid	0.0005
Pyruvic acid	0.0126	Betaine	0.0009
Threonine	0.0284	Creatinine	0.0013
Citric acid	0.0343	Formic acid	0.0013
Phenylalanine	0.0413	Acetone	0.0013
Glutamine	0.0494	Pyruvic acid	0.0031
Acetic acid	0.0588	myo-Inositol	0.0041
Histidine	0.0821	Glutamine	0.0054
Acetone	0.0961	Dimethyl sulfone	0.0071
Alanine	0.0963	Allantoin	0.0071
Dimethyl sulfone	0.0963	Fumaric acid	0.0091
Tyrosine	0.1124	Histidine	0.0118

Sex		Year	
Metabolite	p-value	Metabolite	p-value
Betaine	0.1124	Alanine	0.0152
Fumaric acid	0.1208	Threonine	0.0193
Allantoin	0.1306	Mannose	0.0216
Formic acid	0.1509	Tyrosine	0.0243
3-Hydroxyisobutyric acid	0.1988	Leucine	0.0305
Leucine	0.2568	Succinic acid	0.0305
alpha-Glucose	0.2899	Phenylalanine	0.038
Hippuric acid	0.3256	Valine	0.0703
Mannose	0.3443	alpha-Glucose	0.1023
Valine	0.3643	beta-Glucose	0.1223
3-Hydroxybutyric acid	0.4057	Glutamic acid	0.1451
myo-Inositol	0.4057	Isoleucine	0.2332
beta-Glucose	0.4497	3-Hydroxybutyric acid	0.3099
N,N-Dimethylglycine	0.4955	3-Hydroxyisobutyric acid	0.4529
Lactic acid	0.4963	Lactic acid	0.566
Glutamic acid	0.6501	Acetic acid	0.6272
Succinic acid	0.8501	Hippuric acid	0.7573
Isoleucine	0.8798	Creatine	0.8946
Creatine	0.9397	N,N-Dimethylglycine	0.9647

Table B 3: p-values based on pregnancy status and year of serum metabolites in female giraffes. p values based on year include only the giraffes located in Rooiport game reserve.

Pregnancy status		Year	
Metabolite	p-value	Metabolite	p-value
Fumaric acid	0.0814	Formic acid	0.0455

Pregnancy status		Year	
Metabolite	p-value	Metabolite	p-value
Glutamic acid	0.0833	Citric acid	0.0455
alpha-Glucose	0.0833	myo-Inositol	0.0455
Betaine	0.0833	Allantoin	0.0956
beta-Glucose	0.0833	Pyruvic acid	0.0956
3-Hydroxybutyric acid	0.0833	Tyrosine	0.0956
Isoleucine	0.0833	Dimethyl sulfone	0.0956
Glutamine	0.0833	Creatinine	0.0956
Alanine	0.1489	alpha-Glucose	0.0956
Succinic acid	0.1489	Betaine	0.0956
Acetic acid	0.1489	beta-Glucose	0.0956
Leucine	0.1489	Valine	0.1824
Lactic acid	0.1489	Leucine	0.1824
Valine	0.1489	Acetone	0.1824
Phenylalanine	0.1489	Isoleucine	0.1824
Pyruvic acid	0.1489	Mannose	0.1824
Tyrosine	0.1489	Phenylalanine	0.1824
Threonine	0.2482	Lactic acid	0.1824
N,N-Dimethylglycine	0.2482	Threonine	0.1824
Mannose	0.2482	Histidine	0.1824
Histidine	0.2482	Glutamine	0.1824
Creatinine	0.2482	Fumaric acid	0.2405
Creatine	0.3865	Alanine	0.3173

Pregnancy status		Year	
Metabolite	p-value	Metabolite	p-value
Dimethyl sulfone	0.3865	3-Hydroxybutyric acid	0.3173
Allantoin	0.5637	Succinic acid	0.3173
Formic acid	0.5637	Glutamic acid	0.3173
Citric acid	0.7728	Acetic acid	0.505
3-Hydroxyisobutyric acid	0.7728	3-Hydroxyisobutyric acid	0.505
myo-Inositol	0.7728	N,N-Dimethylglycine	0.505
Acetone	0.7728	Hippuric acid	1
Hippuric acid	0.7728	Creatine	1

Table B 4: p-values based on pregnancy status and year for giraffe milk metabolites. p values based on year include only the giraffes located in Rooiport game reserve.

Pregnancy status		Year	
Metabolite	p-value	Metabolite	p-value
Succinic acid	0.0956	Citric acid	0.0528
Alanine	0.0956	Niacinamide	0.0528
Niacinamide	0.0956	Uridine	0.0528
UDP	0.0956	Lactose	0.0528
Hippuric acid	0.0956	Phosphocholine	0.0528
Lactic acid	0.0956	Succinic acid	0.0528
Isoleucine	0.0956	2-Ketoglutaric acid	0.0528
Valine	0.0956	N-Acetylgalactosamine	0.0528
UDP-N-Acetylglucosamine	0.0956	Creatine	0.0528
Formic acid	0.1824	Pyruvic acid	0.0528
2-Ketoglutaric acid	0.1824	Fumaric acid	0.0528

Pregnancy status		Year	
Metabolite	p-value	Metabolite	p-value
Creatinine	0.1824	UDP	0.0528
Creatine	0.1824	Orotic acid	0.0528
UDP-Glucose	0.1824	Creatinine	0.0528
Orotic acid	0.1824	Glycerophosphocholine	0.0528
Glycerophosphocholine	0.1824	Hippuric acid	0.0528
UDP-Galactose	0.1824	Betaine	0.0528
Taurine	0.1824	UDP-Glucose	0.0528
Acetic acid	0.1824	Butyric acid	0.0528
UDP-N-Acetylgalactosamine	0.1824	UDP-Galactose	0.0528
Uridine	0.3173	UDP-N-Acetylgalactosamine	0.0528
Phenylalanine	0.3173	UDP-N-Acetylglucosamine	0.0528
Fumaric acid	0.3173	3-Hydroxybutyric acid	0.1213
Citric acid	0.3173	N-Acetylglucosamine	0.1213
Butyric acid	0.3173	Caproic/Caprylic acid	0.1213
Caproic/Caprylic acid	0.3173	Taurine	0.1213
Pyruvic acid	0.505	Acetone	0.4386
Betaine	0.505	Alanine	0.4386
Leucine	0.505	Acetic acid	1
Lactose	0.505	Valine	1
N-Acetylglucosamine	0.505	Isoleucine	1
Acetone	0.7389	Lactic acid	1
3-Hydroxybutyric acid	0.7389	Phenylalanine	1
Phosphocholine	1	Formic acid	1
N-Acetylgalactosamine	1	Leucine	1

Table B 5: p-values for giraffe milk nutrients. p values based on year include only the giraffes located in Rooiport game reserve.

Pregnancy status		Year	
Nutrient	p value	Nutrient	p value
Myristoleic acid	0.0429	NPN	0.0424
Elaidic acid	0.0651	Arachidonic acid	0.0445
Nonoadecanoic acid	0.0956	Vaccenic acid	0.0507
Whey	0.1313	Conjugated linoleic acid	0.0507
Ash	0.1824	Heptadecenoic acid	0.0507
Palmitic acid	0.1824	Omega-3 Fatty Acids	0.0528
Total protein	0.1824	α-Linolenic acid	0.0528
Caprylic acid	0.1824	Linoleic acid	0.0528
Total nitrogen	0.2405	Poly Unsaturated Fatty Acids	0.0528
Pentadecylic acid	0.2405	Omega- 6 Fatty Acids	0.0528
% Moisture	0.3173	Na	0.0528
Heptadecenoic acid	0.4018	Lactose	0.0528
Lignoceric acid	0.4972	Oleic acid	0.0528
Eicosatrienoic acid	0.4998	Fe	0.0528
Arachidic acid	0.5024	Margaric acid	0.0528
Conjugated linoleic acid	0.5024	Arachidic acid	0.0528
P	0.505	P	0.0528
Fe	0.505	Mono Unsaturated Fatty Acids	0.0528
Heneicosanoic acid	0.505	Caproic acid	0.0528
Omega-3 Fatty Acids	0.505	Butyric acid	0.0528

Caproic acid	0.505	Pentadecylic acid	0.0528
Ca	0.505	Dry matter	0.0528
Linoleic acid	0.505	Caprylic acid	0.0528
Lactose	0.505	Casein	0.0528
Stearic acid	0.505	% Fat	0.0528
Saturated Fatty Acids	0.505	Lignoceric acid	0.0759
Vaccenic acid	0.615	Eicosatrienoic acid	0.0786
α-Linolenic acid	0.7389	Elaidic acid	0.1213
Myristic acid	0.7389	Mg	0.1213
Palmitoleic acid	0.7389	Stearic acid	0.1213
Dry matter	0.7389	Palmitoleic acid	0.1213
Oleic acid	0.7389	Palmitic acid	0.1213
Mono Unsaturated Fatty Acids	0.7389	Heneicosanoic acid	0.1213
Al	0.7389	K	0.2453
Margaric acid	0.7389	Nonoadecanoic acid	0.2453
% Fat Free Dry Matter	0.7389	Al	0.2453
Casein	0.7389	% Fat Free Dry Matter	0.2453
Capric acid	0.7389	Ca	0.2453
K	0.7389	Myristoleic acid	0.3286
% Fat	1	Total nitrogen	0.3286
Lauric acid	1	Lauric acid	0.4344

Poly Unsaturated Fatty Acids	1	Total protein	0.4386
Butyric acid	1	Capric acid	0.4386
Omega- 6 Fatty Acids	1	Saturated Fatty Acids	0.4386
Mg	1	% Moisture	0.4386
Na	1	Whey	0.6959
Arachidonic acid	1	Ash	0.6985
NPN	1	Myristic acid	0.6985

Table B 6: Comparison of metabolites (µM) in the serum of giraffes and humans.

Metabolite	Giraffe	Human[1]	Metabolic origin[2]
2-Hydroxybutyric acid	-	31.3 ± 7.8	Propanoate metabolism
3-Hydroxybutyric acid	31.77 ± 12.32	76.9 ± 66.3	Fatty Acid Biosynthesis
3-Hydroxyisobutyric acid	2.86 ± 0.99	-	Valine, Leucine and Isoleucine Degradation
D-Glucose	-	4971.3 ± 372.8	Galactose metabolism
Acetic acid	33.06 ± 24.88	41.9 ± 15.1	Fatty Acid Biosynthesis
Acetoacetic acid	-	40.6 ± 36.5	Synthesis and degradation of ketone bodies
Acetone	0.99 ± 0.58	54.4 ± 29.6	Ketone Body Metabolism
Alanine	124.11 ± 32.71	427.2 ± 84.4	Alanine metabolism
Allantoin	16.71 ± 3.70	-	Uric acid metabolism
alpha-Glucose	285.61 ± 123.58	-	Glycolysis or Gluconeogenesis
beta-Glucose	218.94 ± 101.24	-	Glycolysis or Gluconeogenesis
Arginine	-	113.6 ± 14.6	Arginine and proline metabolism
Asparagine	-	82.4 ± 7.3	Alanine, aspartate and glutamate metabolism

Metabolite	Giraffe	Human[1]	Metabolic origin[2]
Aspartic acid	-	20.9 ± 6.1	Alanine, aspartate and glutamate metabolism
Betaine	21.00 ± 5.66	72 ± 22.4	Methionine Metabolism
Carnitine	-	45.7 ± 11.6	Beta Oxidation of Very Long Chain Fatty Acids
Choline	-	14.5 ± 5.3	Glycerophospholipid metabolism
Citric acid	28.39 ± 11.98	114.2 ± 27	Citric acid cycle
Creatine	21.52 ± 7.49	36.7 ± 28.3	Arginine and proline metabolism
Creatinine	17.90 ± 4.78	86.6 ± 18.8	Arginine and proline metabolism
Cysteine	-	33.5 ± 10.3	Cysteine and methionine metabolism
Dimethyl sulfone	8.38 ± 4.59	-	Sulfur metabolism
Formic acid	2.34 ± 1.43	32.8 ± 13.3	Fatty acid metabolism
Fumaric acid	0.80 ± 0.22	-	Citric acid cycle
Glutamic acid	22.78 ± 4.77	97.4 ± 13.2	D-glutamine and D-glutamate metabolism
Glutamine	34.59 ± 10.38	510.4 ± 118.2	D-glutamine and D-glutamate metabolism
Glycerol	-	431.6 ± 100.4	Glycerolipid metabolism
Glycine	-	325.4 ± 126.8	Glycine, serine and threonine metabolism
Hippuric acid	13.16 ± 4.57	-	Phenylalanine metabolism
Histidine	9.22 ± 2.13	131.2 ± 37.3	Histidine Metabolism
Hypoxanthine	-	34.2 ± 10.3	Purine metabolism
Isoleucine	12.86 ± 3.59	60.7 ± 18.6	Valine, Leucine and Isoleucine Degradation
Isopropyl alcohol	-	83.3 ± 132.8	Propanoate metabolism
Lactic acid	1381.39 ± 543.26	1489.4 ± 371.2	Pyruvate metabolism
Leucine	14.54 ± 3.91	98.7 ± 11.5	Valine, Leucine and Isoleucine Degradation
Lysine	11.13 ± 6.50	178.6 ± 58.2	Lysine Degradation
Malonic acid	-	13.5 ± 1.2	Beta-Alanine metabolism

Metabolite	Giraffe	Human[1]	Metabolic origin[2]
Mannose	6.78 ± 1.49	-	Galactose metabolism
Methanol	-	77.4 ± 16.3	Methane metabolism
Methionine	-	29.8 ± 6.3	Cysteine and methionine metabolism
Myo-Inositol	11.38 ± 3.53	-	Inositol phosphate metabolism
N,N-Dimethylglycine	0.48 ± 0.28	-	Methionine metabolism
Ornithine	-	66.9 ± 15.3	Arginine and proline metabolism
Phenylalanine	9.14 ± 1.84	78.1 ± 20.5	Phenylalanine and tyrosine metabolism
Proline	-	198.3 ± 64.8	Arginine and proline metabolism
Propylene glycol	-	22.3 ± 3.3	Glycerolipid metabolism
Pyruvic acid	15.26 ± 6.32	34.5 ± 25.2	Glycolysis
Succinic acid	8.11 ± 2.51	-	Citric acid cycle
Serine	-	159.8 ± 26.6	Glycine, serine and threonine metabolism
Threonine	18.13 ± 7.50	127.7 ± 41	Valine, leucine and isoleucine metabolism
Tryptophan	-	54.5 ± 9.7	Tryptophan metabolism
Tyrosine	6.59 ± 1.60	54.5 ± 9.7	Phenylalanine and tyrosine metabolism
Valine	29.28 ± 8.43	212.3 ± 61.3	Valine, Leucine and Isoleucine metabolism

[1](Psychogios et al., 2011); [2] (Wishart et al., 2007; Wishart et al., 2009; Wishart et al., 2012; Wishart et al., 2018)

Table B 7: Metabolites (µM) in the milk of giraffes compared to Brown Swiss cows and Simmental cows.

Metabolite	Giraffe average	Brown Swiss cows and Simmental cows[1]	Metabolic origin[2]	Giraffe method of detection	Cow method of detection
α-Aminoadipic acid	-	195-427	Lactose biosynthesis	-	GC-MS
α-D-Galactose	-	1200-1760	Lactose biosynthesis	-	NMR
α-D-Lactose	-	45792-61243	Lactose biosynthesis	-	NMR

Metabolite	Giraffe average	Brown Swiss cows and Simmental cows[1]	Metabolic origin[2]	Giraffe method of detection	Cow method of detection
β-D-Lactose	-	75067-99360	Lactose biosynthesis	-	NMR
β-D-Galactose	-	1153-1696	Lactose biosynthesis	-	NMR
β-Hydoxybutyrate	-	10-531	Ketone body production	-	GC-MS
2-Ketoglutaric acid	607.59 ± 242.84	-	Citric acid cycle	NMR	-
3-Hydroxybutyric acid	38.82 ± 69.35	-	Fatty Acid Biosynthesis	NMR	-
3-Methylhistidine	-	103-151	Histidine metabolism	-	NMR
Acetic acid	53.05 ± 58.14	108-701	Fatty Acid Biosynthesis	NMR	NMR
Acetone	11.08 ± 2.83	12-661	Ketone Body Metabolism	NMR	NMR
Alanine	73.42 ± 40.98	-	Alanine, aspartate and glutamate metabolism	NMR	-
Aspartate	-	10-77	Alanine, aspartate and glutamate metabolism	-	GC-MS
Betaine	217.72 ± 112.82	459-1410	Methionine Metabolism	NMR	NMR
Butyric acid	525.64 ± 554.83	-	Fatty acid metabolism	NMR	-
Caproic/Caprylic acid	704.73 ± 716.11	-	Fatty acid metabolism	NMR	-
Carnitine	-	346	Beta Oxidation of Very Long Chain Fatty Acids	-	NMR
Choline	-	150-997	Glycerophospholipid metabolism	-	NMR
Citric acid	7607.51 ± 2572.69	3026-9854	Citric acid cycle	NMR	GC-MS
Creatine	971.55 ± 372.85	-	Arginine and proline metabolism	NMR	-
Creatinine	1439.73 ± 604.78	80-167	Arginine and proline metabolism	NMR	NMR

Metabolite	Giraffe average	Brown Swiss cows and Simmental cows[1]	Metabolic origin[2]	Giraffe method of detection	Cow method of detection
D-Glucose	-	76-662	Glucose synthesis	-	GC-MS
Ethanolamine	-	323	Glycerophospholipid metabolism	-	NMR
Formic acid	10.72 ± 4.01	-	Fatty acid metabolism	NMR	-
Fumaric acid	37.61 ± 19.71	2-81	Citric acid cycle	NMR	GC-MS
Glutamate	-	44-693	D-Glutamine and D-glutamate metabolism	-	GC-MS
Glycerophosphocholine	418.03 ± 209.46	284-1460	Lipid metabolism pathway	NMR	NMR
Glycine	-	33-1109	Glycine, serine and threonine metabolism		GC-MS
Hippuric acid	141.53 ± 84.47	-	Phenylalanine metabolism	NMR	-
Isoleucine	19.48 ± 14.45	2-13	Valine, Leucine and Isoleucine Degradation	NMR	GC-MS
Lactic acid	898.52 ± 945.87	2-3538	Glycolysis	NMR	GC-MS
Lactose	96894.17 ± 22331.92	118186-160121	Lactose Synthesis	NMR	NMR
Leucine	41.51 ± 29.08	2-22	Valine, Leucine and Isoleucine Degradation	NMR	GC-MS
Lysine	-	7-102	Lysine degradation and Lysine biosynthesis	-	GC-MS
Malate	-	8-441	Citric acid cycle	-	GC-MS
N-Acetylcarbohydrates	-	1135-4240	Carbohydrate metabolism	-	NMR
N-Acetylgalactosamine	207.89 ± 46.43	-	Galactose metabolism	NMR	-
N-Acetylglucosamine	261.57 ± 163.59	-	N-acetylglucosamine degradation	NMR	-
Niacinamide	11.28 ± 4.15	-	Nicotinate and Nicotinamide Metabolism	NMR	-

Metabolite	Giraffe average	Brown Swiss cows and Simmental cows[1]	Metabolic origin[2]	Giraffe method of detection	Cow method of detection
Ornithine	-	2-15	Glycine, serine and threonine metabolism	-	GC-MS
Orotic acid	31.64 ± 13.88	-	Pyrimidine Metabolism	NMR	-
Phenylalanine	37.56 ± 15.44	2-10	Phenylalanine and tyrosine metabolism	NMR	GC-MS
Phosphocholine	439.55 ± 122.71	143-1355	Phospholipid Biosynthesis	NMR	NMR
Phosphocreatinine	-	585-2567	Arginine and proline metabolism	-	NMR
Proline	-	11-47	Arginine and proline metabolism	-	GC-MS
Pyruvic acid	54.35 ± 18.43	2-188	Glycolysis	NMR	GC-MS
Succinic acid	90.04 ± 34.02	16-106	Citric acid cycle	NMR	GC-MS
Taurine	1209.99 ± 623.15	327-621	Taurine and Hypotaurine Metabolism	NMR	NMR
Threonine	-	1-46	Glycine, serine and threonine metabolism	-	GC-MS
Trimethylamine-N-oxide	-	43-46	Methane metabolism	-	NMR
Tryptophan	-	1-5	Tryptophan metabolism	-	GC-MS
Tyrosine	5.32 ± 11.10	2-11	Phenylalanine and tyrosine metabolism	NMR	GC-MS
UDP	38.03 ± 13.15	-	Various pathways	NMR	-
UDP-Galactose	391.08 ± 441.17	-	Lactose synthesis	NMR	-
UDP-Glucose	341.94 ± 265.93	-	Lactose synthesis	NMR	-
UDP-N-Acetylgalactosamine	35.95 ± 50.51	-	Galactose metabolism	NMR	-
UDP-N-Acetylglucosamine	76.41 ± 82.00	-	Amino Sugar Metabolism	NMR	-
Uridine	240.45 ± 120.97	-	Pyrimidine Metabolism	NMR	-

Metabolite	Giraffe average	Brown Swiss cows and Simmental cows[1]	Metabolic origin[2]	Giraffe method of detection	Cow method of detection
Valine	31.23 ± 23.60	5-29	Valine, Leucine and Isoleucine metabolism	NMR	GC-MS

[1] (Klein et al., 2010); [2] (Wishart et al., 2007; Wishart et al., 2009; Wishart et al., 2012; Wishart et al., 2018)

Table B 8: Comparison of nutrients between the giraffe averages obtained in this study with that of ruminants from literature.

Nutrient	Giraffe average	Non-pregnant lactating giraffes	Pregnant lactating giraffes	Cow[1,2, 4]	Goat [2, 3, 7]	Giraffe[1]
Total protein (g/100g)	3.98 ± 1.06	4.18 ± 1.17	3.37 ± 0.40	3.271	3.42	4.9
Whey (g/100g)	0.82 ± 0.35	0.94 ± 0.28	0.47 ± 0.35	0.631	0.63	1.77
Casein (g/100g)	3.15 ± 1.12	3.24 ± 1.27	2.90 ± 0.76	2.61	2.113	3.14
Fat (g/100g)	6.61 ± 1.60	6.30 ± 1.02	7.69 ± 3.34	3.91	3.82	7.94
Butyric acid (C4:0) (% of total)	0.48 ± 0.16	0.49 ± 0.18	0.45 ± 0.15	2.874	2.034	0.65
Caproic acid (C6:0) (% of total)	1.37 ± 0.31	1.39 ± 0.35	1.31 ± 0.18	2.014	2.784	1.75
Caprylic acid (C8:0) (% of total)	2.15 ± 0.40	2.18 ± 0.45	2.05 ± 0.02	1.394	2.924	2.67
Capric acid (C10:0) (% of total)	6.42 ± 1.15	6.42 ± 1.31	6.40 ± 0.52	3.034	9.594	8.56
Lauric acid (C12:0) (% of total)	1.11 ± 0.10	1.12 ± 0.09	1.10 ± 0.17	3.644	4.524	1.28
Oleic acid (C18:1c9) (% of total)	27.07 ± 3.39	27.45 ± 3.37	25.74 ± 4.38	22.364	18.64	16.91
Lactose(g/100g)	4.57 ± 0.79	4.64 ± 0.90	4.36 ± 0.40	4.81	4.12	4.16
Galactose (g/100g)	0.04 ± 0.04	0.05 ± 0.03	0.00 ± 0.00	0.051	-	-
Oligosaccharides	-	-	-	-	-	-
Ash (g/100g)	1.08 ± 0.30	1.16 ± 0.33	0.88 ± 0.004	0.701	0.862	-
Calcium (mg/100g)	241.71 ± 61.38	218.24 ± 56.93	300.36 ± 3.02	1222	1342	-

[1](Osthoff et al., 2017); [2](Park, 2016); [3](Kumar et al., 2012); [4](Markiewicz-Keszycka et al., 2013

PCA Pots

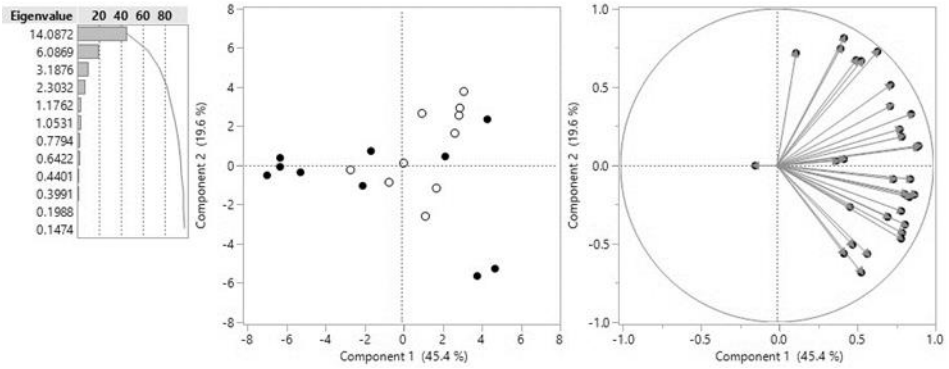


Figure B 1: Principal component analysis (PCA) plot for giraffe serum metabolites based on sex. Closed circle = Female. Open circle = Male.

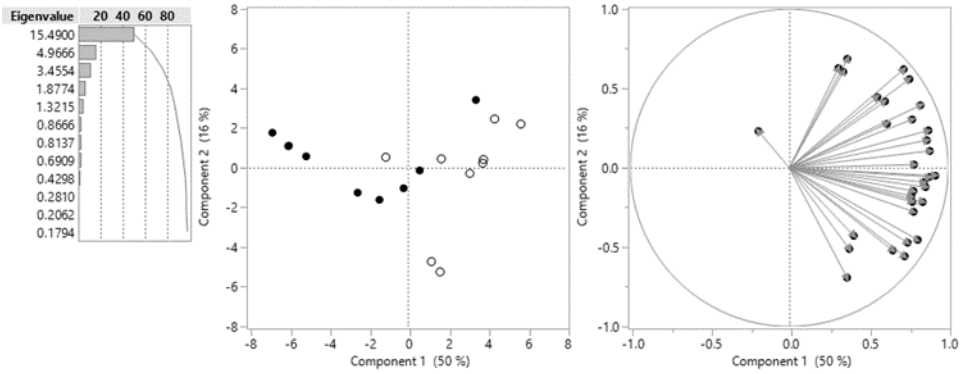


Figure B 2: Principal component analysis (PCA) plot for male and female Rooiport giraffe serum metabolites based on year. Closed circle = 2017. Open circle = 2018.

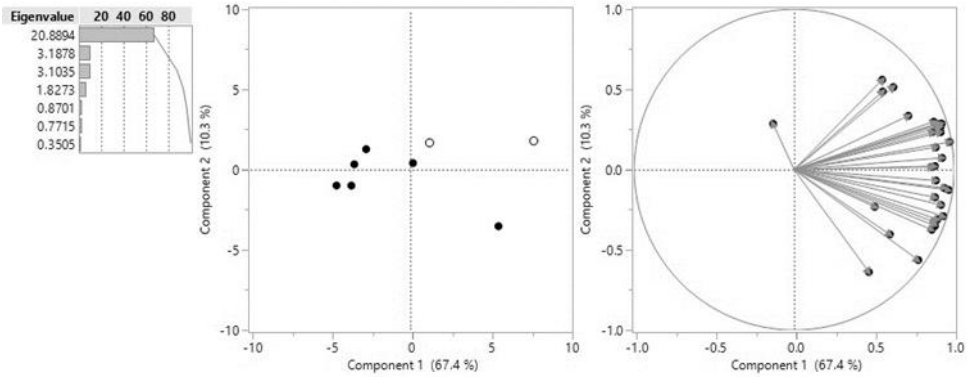


Figure B 3: Principal component analysis (PCA) plot for Rooiport giraffe serum metabolites based on year for females. Closed circle = 2017. Open circle = 2018.

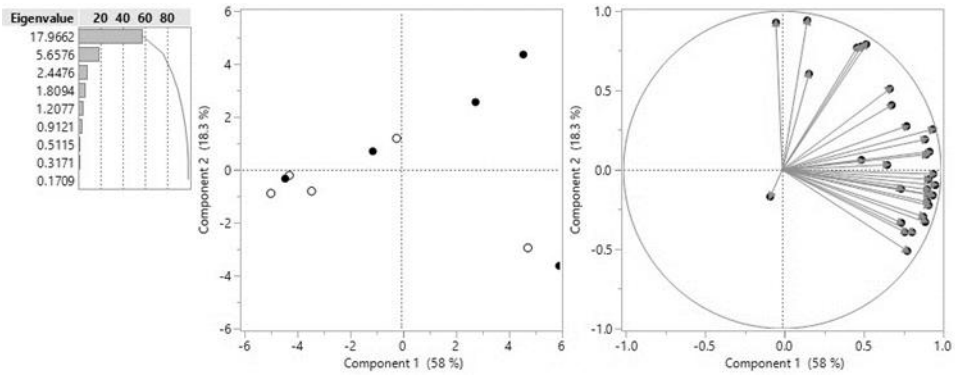


Figure B 4: Principal component analysis (PCA) plot for giraffe serum metabolites based on pregnancy status. Closed circle = non-pregnant. Open circle = pregnant.

Figure B1 is a principal component analysis (PCA) plot showing that there is no separation based on sex for giraffe serum metabolites. The PCA bi-plot of the animals and the metabolites explains 65% of the variation, of which component 1 explains 45.4% and component 2, 19.6%.

Figure B2 is a PCA plot showing that there is no separation based on year for serum metabolites for the male and female giraffes located in Rooiport. The PCA bi-plot of the animals and the metabolites explains 66% of the variation, of which component 1 explains 50% and component 2, 16%.

Figure B3 is a PCA plot showing that there is no separation based on year for serum metabolites for the female giraffes located in Rooiport. The PCA bi-plot of the animals and the metabolites explains 77.7% of the variation, of which component 1 explains 67.4% and component 2, 10.3%.

Figure B4 is a PCA plot showing that there is no separation between the pregnant lactating and non-pregnant lactating giraffe serum metabolites. The PCA bi-plot of the animals and the metabolites explains 68.3% of the variation, of which component 1 explains 50% and component 2, 18.3%.

The eigenvalues show how much variation there is in the data. The smaller eigenvalues do not explain much of the data and so it is only useful to look at the first two components to identify separation between the groups of giraffes. Component 1 of the PCA plot explains the largest portions of the variation.

The fact that there are only two female individuals of 2018 from Rooiport reserve and the overall small giraffe sample size makes a proper statistical analysis impossible. Only a possible trend can be described.

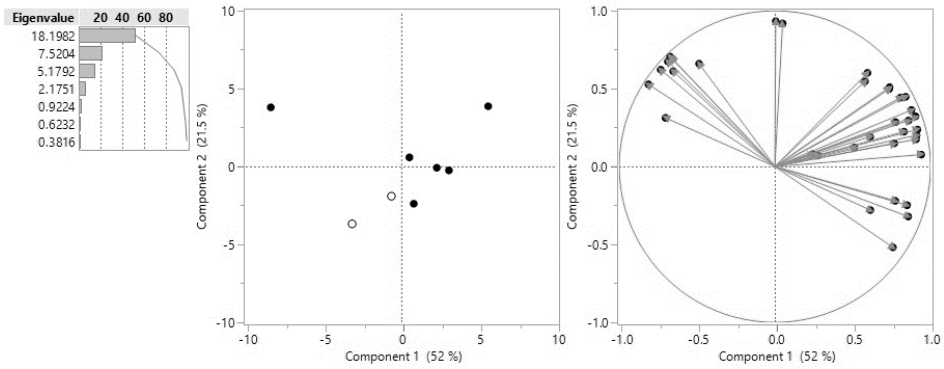


Figure B 5: Principal component analysis (PCA) plot for giraffe milk metabolites based on pregnancy status. Closed circle = non-pregnant. Open circle = pregnant.

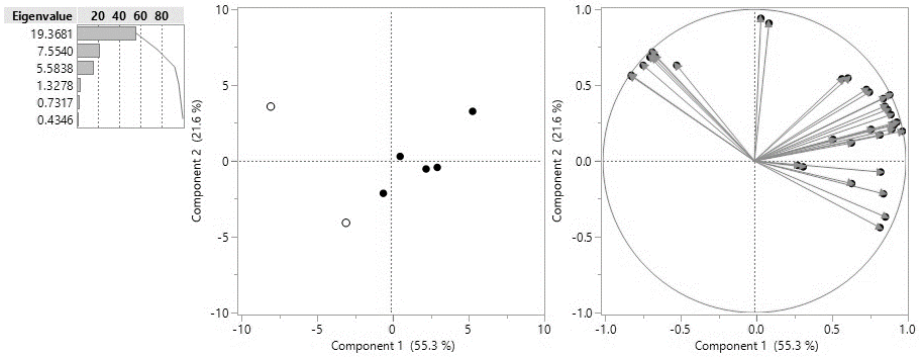


Figure B 6: Principal component analysis (PCA) plot for Rooiport giraffe milk metabolites based on year. Closed circle = 2017. Open circle = 2018.

Figure B5 is a principal component analysis (PCA) plot showing that there is no separation based on pregnancy status for milk metabolites. The PCA bi-plot of the animals and the metabolites explains 73.5% of the variation, of which component 1 explains 52% and component 2, 21.5%.

Figure B6 is a PCA plot showing that there is no separation based on year for milk metabolites for the female giraffes located in Rooiport. The PCA bi-plot of the animals and the metabolites explains 76.9% of the variation, of which component 1 explains 55.3% and component 2, 21.6%.

The eigenvalues show how much variation there is in the data. The smaller eigenvalues do not explain much of the data and so it is only useful to look at the first two components to identify separation between the groups of giraffes. Component 1 of the PCA plot explains the largest portions of the variation.

The fact that there are only two female individuals of 2018 from Rooiport reserve and only two pregnant individuals and the overall low giraffe sample size makes a proper statistical analysis impossible. Only a possible trend can be described.

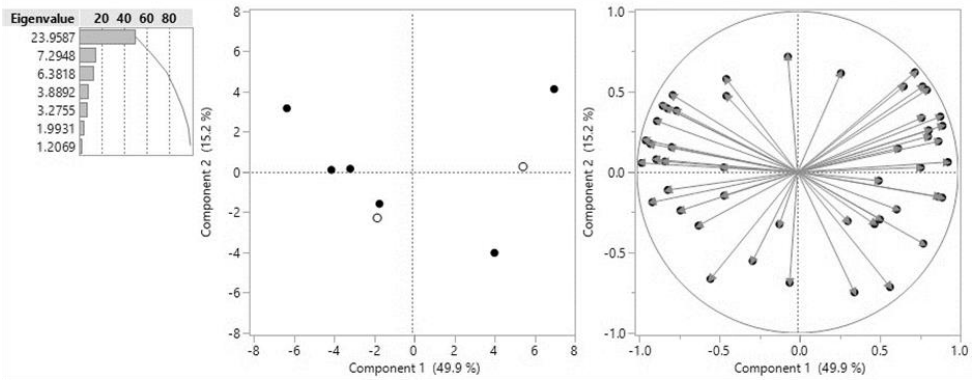


Figure B 7: Principal component analysis (PCA) plot for giraffe milk nutrients based on pregnancy status. Closed circle = non-pregnant. Open circle = pregnant.

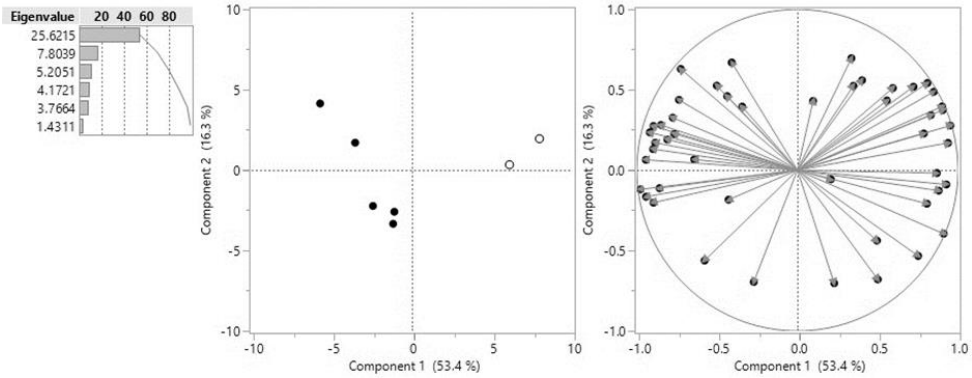


Figure B 8: Principal component analysis (PCA) plot for Rooiport giraffe milk nutrients based on year. Closed circle = 2017. Open circle = 2018.

Figure B7 is a principal component analysis (PCA) plot showing that there is no separation based on pregnancy status for the milk nutrients. The PCA bi-plot of the animals and the metabolites explains 65.1% of the variation, of which component 1 explains 49.9% and component 2, 15.2%.

Figure B8 is a PCA plot showing that there is separation based on year for the milk nutrients of the female giraffes located in Rooiport. The PCA bi-plot of the animals and the metabolites explains 69.7% of the variation, of which component 1 explains 53.4% and component 2,

16.3%. A clustering of the markers for the 2017 group and a clustering of the markers for the 2018 group shows that there are some differences between the two groups.

The eigenvalues show how much variation there is in the data. The smaller eigenvalues do not explain much of the data and so it is only useful to look at the first two components to identify separation between the groups of giraffes. Component 1 of the PCA plot explains the largest portions of the variation.

The fact that there are only two female individuals of 2018 from Rooiport reserve and only two pregnant individuals and the overall low giraffe sample size makes a proper statistical analysis impossible. Only a possible trend can be described.

Chapter 9 – Appendix C

Weather data

Table C1 and C2 show the rainfall received at weather stations surrounding Rooiport Nature Reserve in 2017 and 2018. The rainfall from all the stations was averaged in order to determine the possible amount of rainfall received by the reserve. The amount of rainfall one month prior to the sampling dates was also included to account for new immediate plant growth which the giraffes would then consume. Samples were taken from the giraffes on 1st October 2017 and again on 4th November 2018. The stations included Kimberly, Vaalharts, Barkly West, and Delportshoop. Year 2018 (319.28mm) received less rainfall than year 2017 (370.35mm). Seasonal rainfall data was also included to account for seasonal plant growth. Year 2017 was part of a series of dry years, and 2018 continued to be dry.

Table C3 and C4 show the average temperatures (°C) at weather stations surrounding Rooiport reserve in 2017 and 2018. The temperature data was also included to determine the possibility of extreme temperatures placing stress on the giraffes and therefore affecting the the serum metabolite results.

Table C 1: Rainfall (mm) at weather stations surrounding Rooiport reserve in 2017

Stations	Year average[1]	Five months prior[2]	One month prior[3]	Winter[4]	Spring[5]
Kimberley	361	9	6	0	37
Vaalharts	443	38	26	0	73
Barkly West	343.4	37.5	36.5	0	55.9
Delportshoop	334	0	0	0	0
Average	370.35	21.13	17.13	0	41.48

[1] 1st October 2016 to 1st October 2017
[2] 1st May 2017 to 1st October 2017
[3] 1st Septemeber 2017 to 1st October 2017
[4] 1st June 2017 to 31st August 2017
[5] 1st September 2017 to 30th November 2017
(South African Weather Service, 2019)

Table C 2: Rainfall (mm) at weather stations surrounding Rooiport reserve in 2018

Stations	Year average[1]	Five months prior[2]	One month prior[3]	Winter[4]	Spring[5]
Kimberley	294	5	2	0	20
Vaalharts	480	15	6	8	9
Barkly West	328.9	12.7	4	6.5	8.7
Delportshoop	174.2	9.2	6	3.1	6.1

Stations	Year average[1]	Five months prior[2]	One month prior[3]	Winter[4]	Spring[5]
Average	319.28	10.48	4.5	4.4	10.95
[1]1st October 2017 to 4th November 2018 [2]1st June 2018 to 4th November 2018 [3]1st October 2018 to 4th November 2018 [4] 1st June 2018 to 31st August 2018 [5] 1st September 2018 to 30th November 2018 (South African Weather Service, 2019)					

Table C 3: Average temperatures (°C) at weather stations surrounding Rooiport reserve in 2017

Stations	September Min	September Max	October Min	October Max	1st October Min	1st October Max
Kimberley	8.4	29.5	9.3	27.6	11.7	27.7
Postmasburg	8.8	28.8	9.4	26.8	16.1	29.1
Vaalharts	8.1	29.8	9.5	27.9	14	27.7
Average	8.4	29.4	9.4	27.4	13.9	28.2
(South African Weather Service, 2019)						

Table C 4: Average temperatures (°C) at weather stations surrounding Rooiport reserve in 2018

Stations	October Min	October Max	November Min	November Max	4th November Min	4th November Max
Kimberley	12.2	31	14.5	33.3	16.1	33.5
Postmasburg	14.4	32.4	15.8	34.8	17.1	32.9
Vaalharts	10.6	30.9	13.2	33.2	15.2	32.5
Average	12.4	31.4	14.5	33.8	16.1	33.0
(South African Weather Service, 2019)						