Gene expression profiles in the Longissimus dorsi muscle of Bonsmara and Nguni cattle fed two different diets

by

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Declaration

I, Dina Alida Linde hereby declare that this thesis, submitted for the MSc(Agric) Animal Science: Animal Breeding and Genetics degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

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In memory of my uncle, Andries Linde (my Pretoria Pa) and my grandfather, Lou Linde.
'The Lord gave and the Lord has taken away; praise the name of the Lord!'
Job 1:21

To the Creator of all things, praised be your name. This thesis glorifies the intricate and complex and amazing way You, Great God, designed life. I hope it is a testament to Your power and Your love. For how wonderful you made this Earth! That one small thing, be it the energy level in nutrition or the environment of the dam, can change the phenotype of the animal. You created this life so that every little detail can praise your name. I hope that I did Your Great Design justice and that Your presence can be felt throughout this whole book, for you were with me with every word I wrote. You gave me my intelligence, may I use it to praise you and the nature You gave us as a sign of Your love.

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‘Those who sow with tears will reap with songs of joy.
Those who go out weeping, carrying seed to sow,
Will return with songs of joy, carrying sheaves with them.’
Psalm 126: 5-6.

Abstract

In South Africa, Nguni cattle are one of the breeds found predominantly in extensive production systems. In this study the effect of two feedlot diets with different energy levels have been investigated using a transcriptome approach. Twenty Nguni and twenty Bonsmara bulls were fed a low or a high energy diet for 120 days and growth parameters were measured. At slaughter, *L. dorsi* muscle samples were collected for transcriptome analysis. Performance results showed a higher live weight, carcass weight and marbling score for all bulls fed the high energy diet compared to bulls fed the low energy diet. Diet had a greater effect on the Bonsmara compared to the Nguni according to transcriptomic and phenotypic values. A total of 73 differentially expressed genes were observed between the diets across breeds. The genes that were involved in intramuscular fat deposition (CRHR2, NR4A3, MMD) were expressed on a higher level in the bulls on the low energy diet compared to bulls on the high energy diet. Genes that were involved in muscle deposition (PITX2, Leptin, AVP) was expressed higher in the bulls on the high energy diet. Comparing the breeds revealed that 2214 genes were differentially expressed between the Bonsmara and the Nguni. At the end of the feedlot trial a higher expression of marbling genes (SIRT, ND, ADIPOQ) were observed in the Nguni, however this expression was not observed in the marbling scores recorded. Several genes (ASIP, MOGAT, SNAI3) that were involved in fat deposition were upregulated in the Bonsmara. This suggests that the Nguni was still growing at the end of the feedlot trial while the Bonsmara had reached physiological maturity. An extended feedlot period for Nguni cattle should be considered in future studies. This study provides reference data for differentially expressed genes in muscle of South African feedlot cattle.

Contents

List of Abbreviations

A	Adenosine
ACSL	Acyl-CoA synthetase
ACTA	Actin, alpha
ADG	Average daily gain
ADIPOQ	Adiponectin
AMPK	Adenosine monophosphate-activated protein kinase
ARC	Agricultural Research Council
API	Animal Production Institute
ASIP	Agouti signalling protein
ATP	Adenosine triphosphate
AVP	Arginine vasopressin-neurophysin
C	Cytosine
CALM	Calmodulin
CAPN	Calpain
CAT	Catalase
cDNA	complementary DNA
CHRND	Acetylcholine receptor delta
CLA	Conjugated linoleic acid
COL	Collagen
COX	Cytochrome C oxidase
CRHR	Corticotropin-releasing factor receptor
CV	Coefficient of variation
CYB	Cytochrome B
DGAT	Diacylglycerol
DM	Dry matter
DNA	Deoxyribonucleic Acids
DNMT	DNA methyltransferase
EMA	Eye muscle area
EST	Electronic Sequencing Tag
FABP	Fatty acid binding protein
FADS	Fatty acid desaturase gene
FAO	Food and Agriculture Organisation of the United Nations
FCR	Feed conversion ratio
FE	Feed efficiency

FOX	Forkhead transcription factor
g	Grams
G	Guanine
GB	Gigabyte
GLM	General Linear Module
GPX	Glutathione Peroxidase
GSTA	Glutathione s-transferase alpha
GTF	General Transcription Factor
HEB	Bonsmara's that received the high energy diet
HEN	Nguni's that received the high energy diet
IGF	Insulin-like Growth Factor
IGFBP	IGF binding protein
IMF	Intramuscular fat
INSIG	Insulin induced gene
IRS	Insulin receptor substrate
kg	Kilogram
LEB	Bonsmara's that received the low energy diet
LEN	Nguni's that received the low energy diet
LSMean	Least square mean
Lys	Lysine
MAPK	Mitogen activated protein kinase
MDC	Malonyl-CoA corboxylase
MDH	Malate dehydrogenase
ME	Metabolizable Energy
MEF	Myocyte Enhancer Factor
MJ	Mega Joules
mg	Milligram
mm	Millimetres
MMP	Matrix metalloproteinase
ml	Millilitre
MOGAT	Monoacylglycerol acyltransferase
MRF	Muscle Regulatory Factors
mRNA	messenger Ribonucleic Acid
MT	Mitochondrial
N	Nitrogen
n	Number

NA	Not applicable
ND	NADH-ubiquinone oxidoreductase
NEFA	Non-esterified Fatty Acids
NGS	Next Generation Sequencing
NPN	Non-protein Nitrogen
NR	Nuclear receptor
NS	Non significant
OXT	Oxytocin neurophysin
PacBio	Pacific Biosciences
PCR	Polymerase Chain Reaction
PGM	Personal Genome Machine
PITX	Pituitary (paired) homeobox transcription factor
POMC	Pro-opiomelanocortin
PPARG	Peroxisome Proliferator Activated Receptor Gamma
PPARGC1A	PPARG Coactivator 1 alpha
PPM	Protein phosphatase
PRKAG	5'-AMP-activated protein kinase Gamma
Proc	Procedure
PSMC	Proteosome
PUFA	Poly Unsaturated Fatty Acids
QC	Quality Control
QTL	Quantitative trait loci
R	Rand
RNA	Ribonucleic Acid
RNA-Seq	Ribonucleic Acid Sequencing
RPKM	Reads per kilobase
RPM	Revolutions per minute
rt-PCR	Reverse Transcription Polymerase Chain Reaction
RTU	Real time Ultrasound
SAGE	Serial Analysis of Gene Expression
SAS	Statistical Analysis Software
SCD	Stearoyl-CoA desaturase
SERPIN	Serine incorporator
SIRT	Sirtuins
SMRT	Single molecule real time
SNP	Single Nucleotide Polymorphism

SOLiD	Sequencing by Oligo Ligation Detection
SPCA	Society of Prevention of Cruelty to Animals
SREBP	Sterol regulatory element binding protein
T	Tyrosine
TDN	Total Digestible Nutrients
TEX	Testis-specific protein
TF	Transcription Factor
TNNT	Troponin T
TPI	Triosephosphate isomerase
tRNA	total RNA
TUBB	Tubulin beta
UFA	Unsaturated Fatty Acids
USP	Ubiquinone carboxyl-terminal hydrolase
UTR	Untranslated region
ZMW	Zero-mode waveguides

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Chapter 1: Introduction

1.1 Introduction

Since the domestication of farm animals over 12 000 years ago, they have been used for food production, hides and cultural and religious purposes (Shabtay, 2015). They have evolved into a number of breeds well adapted to various local environments and production systems. These breeds differ in qualitative and quantitative characteristics, including disease resistance, climate adaptation, fertility, meat quality and nutrient requirements. Cattle indigenous to Africa can be classified into three groups namely *Bos taurus*, *Bos indicus* and Sanga types (Rege, 1999). It has been indicated by Makina *et al.* (2014) that *Bos taurus* breeds, such as Angus and Holstein, were clearly separated from South African indigenous breeds (Nguni, Afrikaner) on a genomic level. The Nguni is a tropically adapted *Bos Taurus* Sanga breed, indigenous to Southern Africa and is primarily raised in extensive production systems (Strydom, 2008). The Nguni people migrated along the eastern coast of Africa, settling in Southern Africa with their Nguni cattle. (Scholtz *et al.*, 2011). Many Nguni cattle are kept in communal systems and contribute to food security and the livelihoods of resource poor farmers in South Africa (Mapiye *et al.*, 2009; Strydom *et al.*, 2008). This small framed breed is often used as dams in crossbreeding systems in (Scholtz *et al.*, 2011). They are characterized by their multi-coloured coats, which may show various patterns (white, black, brown, golden yellow, spotty or dappled). Their noses, however, are always well pigmented (black-tipped) and their horns grow in a variety of shapes.

The efficient use of domesticated animals is crucial due to limited arable land, the environmental impact of livestock and the increasing human population (Niemann *et al.*, 2011). Nguni's are well known for their adaptability to the harsh South African climate and tend to be more productive on pasture based systems when compared to exotic breeds (Strydom, 2008). Many Nguni farmers however still attempt to market their weaners to the feedlots, as their veld does not have enough carrying capacity to meet the nutrient requirements of the calves after weaning. Although South Africa has a large proportion of grazable land, factors like inconsistent rainfall, overgrazing and the variation in biomes limit the carrying capacity of the veld. The feedlot provides as alternative offset for Nguni producers. In harsh areas, pure breeding with Sanga cattle may be the only cost-effective production strategy that can be used (Scholtz *et al.*, 2011). In these areas, the demands of exotic breeds or their crossbreds might not be met due to insufficient management or nutrition.

Since the 1970's when commercial feedlots were established in South Africa it became common practise to finish cattle for the market in these feedlots, rather than from grass feeding. The demand for meat was too high to be satisfied with cattle finished from the veld. Later maturing *Bos Taurus* breeds became more popular to the disadvantage of indigenous cattle, which were regarded as inferior (Bester *et al.*, 2001). Feedlots accept cattle at 7-9 months (weaners) or at the most 10-12

months (long weaners) (Strydom, 2008). Presently, the feedlot industry is under increasing pressure to use breeds that are heavier at the same or younger age than in the past. This is the result of a predicted higher demand for meat in the future due to the increasing human population (Cassar-Malek *et al.*, 2008).

The feed of any livestock production system comprises about 60-70% of its cost. If a less expensive diet can be used in the feedlot, it may hold financial benefits, especially for a breed like the Nguni. Reducing input costs (feed, buying price of the calves), increasing output (carcass weight), increasing production efficiency (feed conversion, average daily gain) or increasing the end-product value (meat quality) is a goal of any production system. Comparative nutritional studies, as envisaged in this project, can lead to new developments in production systems that can have a beneficial effect for the farmer.

The development of genomic technology has made it possible to study the animal at a genome level, which provides an opportunity to better understand the underlying genetic and physiological mechanisms involved in the determination of the expressed traits and the influence of the environment on these traits. The field of nutrigenomics refers to the study of the effects of the diet (nutrition) on the genotype of the animal that results in the phenotype of the animal (Bouchard & Ordovas, 2012) and holds the potential to determine the requirements of an animal under various conditions, e.g. disease, production (nutrition), breeding and environmental interaction (Den Hartog & Sijtsma, 2011). It also studies how feedstuffs (nutrients, additives or other compounds) affect genes and gene expression and will result in feeding the animal according to its genetic potential.

Global warming and population growth become challenges to the livestock industry and to effectively overcome these challenges it is essential to characterize the transcriptomes of divergent phenotypes. It is well known that environmental factors can modify gene expression (Foley *et al.*, 2009). These epigenetic changes are moderated at the biochemical level by the chromatin conformation initiated by DNA methylation, histone variants, post-translational modifications of histones, non-histone chromatin proteins and non-coding RNAs (Russel, 2006a). Both genetic and epigenetic controls should be considered when formulating breeding programs or changing environmental conditions as these controls may interfere in the expression of the genes (Tchurikov, 2005). One or two small changes in gene expression can lead to significant changes in the production cost (McNamara, 2015). Identification of epigenetic controls in diverse environments and conditions could lead to more effective management of such effects in developing countries such as South Africa (Scholtz *et al.*, 2014).

Several methodologies have been developed for measuring gene expression, such as RNA-seq, rt-PCR, Northern blotting, SAGE or microarrays (Wickramasinghe *et al.*, 2014). RNA sequencing is a powerful method for quantifying and mapping transcriptomes and gene expression. It has many advantages over other methods due to the ability to quantify all genes present and not exclusively the genes targeted (Marioni *et al.*, 2008). Gene expression is the result of a number of

processes, including transcription and translation, and the regulation of these processes result in the expression of the phenotype of the animal. In these processes, genes can be over-expressed or under-expressed depending on the internal and external factors and this variation in gene expression can therefore be exploited with regard to selection of favourable traits (Cassar-Malek *et al.*, 2008).

1.2 Aim of study

The maintenance nutritional requirements of the medium-sized hardy breeds are modest and their ability to exploit the low-quality forage in their habitat holds potential (Shabtay *et al.*, 2015). Their low weaning weights, poor post weaning growth rates and perceived poor feed efficiency compared to exotic breeds results in Nguni farmers finding it difficult to access feedlot markets, although their adaptability to the feedlot is acceptable (Strydom *et al.*, 2008). They experience price discrimination from the feedlots, especially when compared to calves from exotic or crossbred genotypes (Strydom, 2008). In 2014, it was recorded that there was a R2/kg – R5/kg penalty on indigenous weaners (Dugmore, 2014). This is a serious threat to the sustainability of farming with indigenous breeds and the utilisation of these breeds. Introduction of exotic genetic material contributes to the dilution of indigenous breeds and may result in loss of unique adaptive traits (Rischkowsky & Pilling, 2007). Despite less favourable growth performance and carcass yield of certain Sanga breeds under intensive feeding conditions compared to exotic breeds (Herring *et al.*, 1996 (Tuli); Meisnner & Roux, 1982 (Afrikaner); Phillips & Holloway, 1995 (Tuli)), there is evidence of variation in maturity types among these breeds that can be utilised. Furthermore, certain performance characteristics under feedlot conditions are also found to be independent of breed type (Strydom *et al.*, 2008). The National Beef Cattle Performance Testing Scheme's growth performance tests showed that the feed conversion ratio of the Nguni compared favourably with that of a number of late maturing breeds, while Strydom *et al.* (2008) found the Nguni, Drakensberger and Bonsmara had similar feed conversion ratios. A number of studies indicate that within their own production systems and especially at harsh conditions, local breeds are well positioned to compete with exotic breeds in terms of productivity (Köhler-Rollefson *et al.*, 2009) and lower output is compensated by the lower required inputs (Anderson, 2003; Scarpa *et al.*, 2003). Therefore, a low energy diet may be beneficial to indigenous breeds.

The Northern Cape Department of Agriculture, Land Reform and Rural Development has requested the Agricultural Research Council (ARC) to conduct research on Nguni cattle that may show improved growth performance on a low energy diet compared to a high energy diet. This project will attempt to investigate genes related to growth and performance in Nguni cattle raised in a feedlot system fed two diets using a transcriptome approach (Next-generation sequencing) where gene expression will indicate the levels of different messenger RNAs (mRNA) in cells (Wickramasinghe *et al.*, 2014) involved in the transcription into functional proteins.

The aim of the study was to investigate potential genes associated with muscle growth in Nguni and Bonsmara fed on two different diets, by performing a transcriptome analysis. To achieve the aim the following objectives were set:

1. To perform a feedlot trial where the animals were raised on low and high energy diets
2. Collection of quantitative (growth data, RTU and feed intake) and physiological (blood analysis) data and samples of the *Longissimus dorsi* muscle at slaughter for the RNA extraction (needed for RNA sequencing) and transcriptome analysis.
3. To compare the transcriptome of Nguni and Bonsmara cattle fed a low and a high energy diet.

The Nguni breed has been included in a number of research projects but this will be the first project to study gene expression of growth related genes and animals raised in a feedlot system on different diets in South Africa.

Chapter 2: Literature Review

2.1 Introduction

Transcriptomics is a relatively new field of study focussing on the transcriptome of the cell; and is also referred to as the gene expression of the cell (Morozova *et al.*, 2009). The transcriptome is the complete set, in type and quantity, of transcripts in a cell (Nagalakshmi *et al.*, 2010), while transcriptomics enables the high throughput screening of expressed genes in a specific tissue and can be used to study the development of complex phenotypic traits determined by genetic and environment interactions, e.g. meat quality (Damon *et al.*, 2013). It can also be used to study the biological function of a cell or a tissue. The focus of many transcriptomic experiments is on the detection of differently expressed and co-expressed genes and the construction of gene networks (Pareek *et al.*, 2011) during development or under different physiological and pathological conditions. Transcriptomic research has focussed on the mechanisms controlling gene expression and the impact of biological and external factors on gene expression (e.g. genetic determinants and nutritional factors) in tissues involved in metabolism, reproduction, growth and production traits (Cassar-Malek *et al.*, 2008).

There are a number of techniques that can be used to study gene expression ranging from candidate gene-based studies (Alwine *et al.*, 1977) to microarray technologies that lead to researchers characterizing thousands of transcripts' expression levels in different cell types (Schena *et al.*, 1995). Although many gene expression (transcriptomics) studies in livestock have used microarrays in the last decade, this technology is being replaced by next generation sequencing (NGS) (Wickramasinghe *et al.*, 2014) such as RNA-seq. RNA-seq was developed to analyse gene expression on an inclusive scale, thereby having many advantages over microarrays such as its extensive genomic range and its ability to quantify all genes present (Marioni *et al.*, 2008).

To understand the control of macronutrient utilization, the transcriptomic changes in productive tissues that occur in response to dietary influences must firstly be known (Baldwin *et al.*, 2012). This can lead to improvements in nutrient use efficiency and product quality. Most traits are complex because they are under the control of an interacting network of genes, each with a small effect, and of environmental factors, such as nutrition (Wu & Lin, 2006). The level of expression of key enzymes is affected by transcriptional control of the metabolic regulation in complex organisms (Desvergne *et al.*, 2006).

The aim of this section is to review recent literature on the principles of transcriptomics with reference to the methodology available, the role of nutrigenomics and the application thereof in beef cattle.

2.2 The principles of gene expression

The phenotype of an organism does not only depend on a change in the gene sequence, but also on a change in the expression of the gene. The expression of the gene may vary due to a number of factors that influence the transcription or translation processes or be due to posttranslational modifications (Russell, 2006a). Gene expression is the result of DNA (genes) that is transcribed into proteins that determine the phenotype of the organism. There are different cell types within an organism each with a different set of genes that are activated or expressed (transcriptome), which leads to different cell functions (Morozova *et al.*, 2009). The patterns of gene expression govern the cellular morphology, development and function (Shlyueva *et al.*, 2014).

The process of transcription where DNA is transcribed to RNA has been well documented in literature (Russell, 2006a) and only a brief outline is provided here. Transcription commence within a region of the DNA strand that is unwound (Figure 2.1) where RNA polymerase binds to the promoter region of the DNA strand to begin with transcription (Klug *et al.*, 2009). RNA polymerase then selects the next nucleotide to add to the chain by pairing to the exposed nucleotide base on the DNA template strand. After the DNA is transcribed to mRNA a terminator stops the process. The RNA transcript (pre-mRNA) is modified in the nucleus by RNA processing to result in mature mRNA. The pre-mRNA consists of introns (not translated) and exons (which are translated). The pre-mRNA is converted to mRNA when the introns are removed. The rate at which transcription is initiated differs from gene to gene. This difference can lead to different genes having different levels of expression (Russell, 2006a). Nutrition can modify the expression of a few transcription factors and thus alter the phenotype by changing a large number of metabolic and developmental pathways (Burdge *et al.*, 2007).

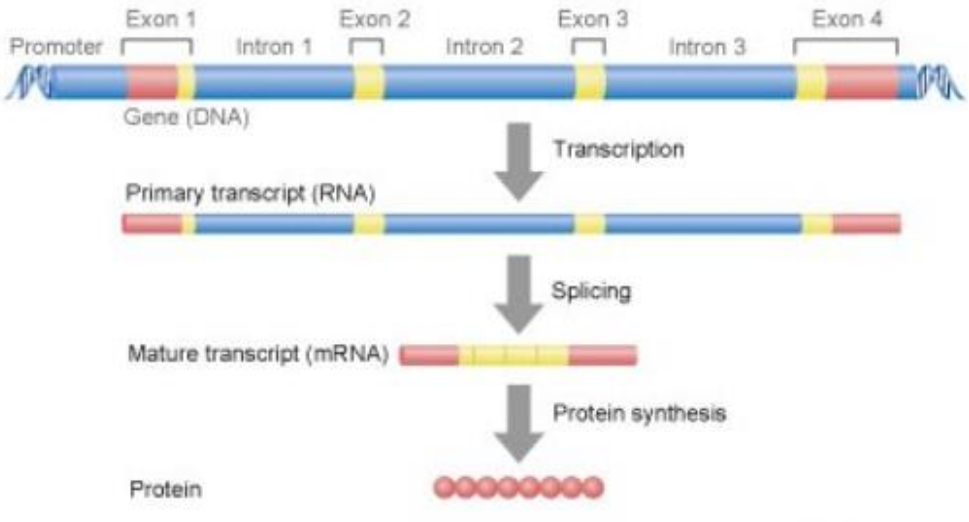

Figure 2.1 The process of transcription and translation that result in proteins that are expressed as the phenotype.

There are several factors that can change the level of expression (Russell, 2006c). An important consideration in understanding the responsible mechanism for phenotype changes is the interaction between any process resulting in different phenotypes, environmental cues and gene polymorphisms, especially those located in gene promoters (Burdge *et al.*, 2007). A mutation in the promoter region can decrease the initiation of transcription that may result in a decrease in the expression of the genes. There are molecules called enhancers which can maximize the transcription process which results in an increase in gene expression (Shlyueva *et al.*, 2014). Enhancer sequences contain short DNA motifs that act as sequence-specific transcription factor binding sites. Enhancers (also known as *cis*-regulatory modules) can be found upstream or downstream from the initiation site (Latchman, 2010). They act independently of the distance and orientation of their target genes (Shlyueva *et al.*, 2014). Gene expression can also be changed with RNA editing (Russell, 2006c). RNA editing is the posttranscriptional insertion or deletion of a nucleotide or the conversion of one nucleotide base pair to another nucleotide base pair. There are three classes of proteins involved in transcription activity; the general transcription factors, the activators and the repressors. The general transcription factors (GTF) does not influence transcription initiation. The activators and co-activators can lead to the activation of transcription by interacting with transcription factors and the repressors counteract the activators and blocks transcription.

The regulation of gene expression takes place in the transcription process (Russell, 2006a) where the maximum expression depends on the regulatory protein (activators) binding to the promoter and enhancer levels (Latchman, 2010). The binding leads to recruitment of the protein required to make chromatin accessible to the transcription machinery (e.g. the enzymes). The regulatory promoter element is specific to the gene it controls as it binds the signalling molecules involved in the regulation of the gene's expression under different conditions. The specificity of the regulatory protein in binding to the regulatory element in the DNA ensures strict control of which genes are turned off and which turned on. Some regulatory proteins activate transcription when it binds to the enhancer or promoter element, others repress transcription (Klug *et al.*, 2009). The net effect of the regulatory proteins on transcription is the combination of the different proteins bound to the strand. If a repressor element binds to an enhancer element the result will depend on the interaction between the two elements. If the repressor is strong, the gene will be silenced, and this type of repressor is called a silencer element. Combining a few regulatory proteins in certain ways will regulate transcription of different genes and large numbers of cell types can be specified, which is called combinatorial gene regulation.

Messenger RNA (mRNA) is converted to protein during translation (Russell, 2006b) when the codon (three series nucleotide) is read by the translation machinery to assemble the correct polypeptide chain bound by a peptide bond. If a single base pair is deleted or added it will result in a different sequence and a different protein. Translation is initiated by the mRNA binding to the

ribosome and the specific tRNA initiator. Aminoacyl-tRNA synthase binds to the ribosome so the peptide bond will form. The ribosome then moves along the mRNA one codon at a time where the elongation will continue until the polypeptide encoded for the mRNA is complete by recognizing the stop codon (Klug *et al.*, 2009).

Posttranslational control takes place when the translation process is complete by regulating the protein product (Russel, 2006a). RNA processing control regulates the production of mRNA molecules from pre-mRNA molecules. There are situations where alternative polyadenylation sites and alternative splicing produce different pre-mRNA and different functional mRNA. The products of alternative polyadenylation or splicing are encoded by the same gene, but differ in structure and in function. The products of mRNA are translated to yield the precursor polypeptide from which the functional hormones are generated posttranslationally by protease cleavage. The regulatory signals change the stability of the mRNA (Klug *et al.*, 2009). The addition of a regulatory molecule to the cell can lead to an increase in the synthesis of certain proteins. This is the result of increasing the rate of transcription of the genes involved or of an increase in the stability of the produced mRNAs. If the mRNA is produced continuously it will also be continuously translated. The level of protein produced is controlled by the rate of the protein degradation (Russel, 2006a).

The regulatory mechanisms for regulating gene function complexes determine the up/down regulation of the genes and the scope of the gene respond to the activation of different signalling pathways (Latchman, 2010). The genomic sequence of an animal plays only a partial role in the phenotype of the animal. For example; a combination of genes and the environment determine the animal's resistance to stress. There are various environmental and nutritional factors that can lead to a change in the phenotype. This is called phenotypic plasticity (Li, 2015). The diversity in phenotypic plasticity in reference to morphology and functionality leads to different phenotypes. These different phenotypes are determined by cell-specific patterns of gene expression controlled by the regulatory sites in the genome. RNA interference is another factor causing variation in gene expression (Russel, 2006c) that may occur when a small fragment of double-stranded RNA with a sequence match of a part of the gene's sequence, interferes with the expression of the gene. Mature mRNA is usually the target for RNA interference when the double stranded RNA has matching exons the genes will not be expressed. This does not, however, happen with matching promoters or introns. The goal of RNA interference is blocking the expression of foreign genes.

2.3 Post translational regulation

Post translational regulation is performed in various processes, such as chromatin regulation, histone modification and DNA methylation. Chromatin regulates the transcription process based on the modification of DNA and of the histone (Li, 2015). It is composed of DNA, various modified histones and non-histone proteins that have an impact on the differentiation of cells, gene regulation

and other processes. Chromatin controls the access to sites that is necessary for the initiation of transcription, however it can have a repressive effect on gene expression because the access to the transcription machinery is physically limited (Shlyueva *et al.*, 2014).

Histones are subject to a wide variety of posttranslational modifications; Lysine acetylation, Lysine and Arginine methylation, Serine and Threonine phosphorylation (Triantaphyllopoulus *et al.*, 2016). These histone modifications are thought to affect the chromosome function through two processes. The one process is when the modifications may alter the electrostatic charge of the histone which results in a structural change in the histones or binding to the DNA (Li, 2015). The condensed chromatin structure is transformed to the relaxed structure which is associated with an increase in the transcription rate. The second process affects the chromosome through the modifications of the binding sites for protein recognition models which recognize acetylated/methylated Lys. The functional protein recruitment may activate or repress the outcome on gene expression. Short chain fatty acids, particularly butyrate, can regulate cell differentiation, growth, motility and can induce the cell cycle and cell death. DNA replication can be inhibited by butyrate treatment by altering the histone methylation to be a histone deacetylase inhibitor (Li, 2015).

DNA methylation is the process where an extra methyl group is transferred to the C5 position of the cytosine (Li, 2015; Tian, 2012). Differentiated cells develop a stable and unique DNA methylation pattern that regulates the tissue-specific gene transcription. When the methylation occurs within the gene's regulatory elements (promotors, enhancers, insulators and repressors) it will suppress the function of the gene (Triantaphyllopoulus *et al.*, 2016). DNA methylation patterns undergo epigenetic reprogramming, meaning it undergoes establishment, re-establishment and maintenance (Li, 2015). There are many nutritional components that can influence DNA and histone methylation. Methyl donor nutrients that are coenzymes of 1-carbon metabolism can modify 1-carbon metabolism. Any nutrients that can modify the activity of DNA methyltransferases can affect DNA and histone methylation (Li, 2015), for example a deficiency of dietary folate.

2.4 Epigenetics & Nutrigenomics

The first description of epigenetics date back to Waddington (1942) where he described epigenetics as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being". Epigenetics can also be described as the temporal and spatial control of gene activity during development of complex organisms (Holliday, 1990). It was Riggs & Porter (1996) who modified the concept to refer to epigenetics as the mitotically heritable changes in gene expression that occur without any changes in the DNA sequence. Gigli & Maizon (2015) refer to it as the changes in gene expression that occur without modification in DNA sequences that can be passed along by mitosis. There are clearly a number of definitions for epigenetics, but all have in common: the change in gene expression, not the DNA sequence and

reference to the effect to be passed on to the next generation. Gene silencing can be used as an example, which is when epigenetic changes are associated with turning off a gene in certain tissues where its expression is no longer required (Goddard & Whitelaw, 2014).

Gene expression is not only changed by the regulatory mechanisms in the cell of an organism but also by factors outside the cell system including temperature, humidity and forage availability. The nutrition of the animal and physiological state of the animal also have an influence on the expression of certain genes (Cassar-Malek *et al.*, 2008). Gene expression can be used to explain the variation found in different phenotypes observed within and between breeds and populations. Finding the balance between these factors that influence the expression of phenotypes holds the potential for sustainable precision livestock farming if animals with extreme phenotypic and favourable characteristics can be compared and used in selection programs (Hocquette *et al.*, 2012; McNamara, 2015). A significant challenge in livestock breeding is to track epigenetic information that changes from one generation to the next (Triantaphyllopoulus *et al.*, 2016).

Epigenomics involves studying the phenomenon of changes in regulation of gene expression and can be divided into many categories (Li, 2015) that depend on the factor that influence the gene's expression. The cells' information is inherited by the next generation through genetic and epigenetic routes. Epigenetics can provide information relating to the heritability of complex traits as well as diseases (Triantaphyllopoulus *et al.*, 2016). Genetic information is encoded in the DNA sequence, while epigenetic information (epigenome) is defined by DNA modification (DNA methylation) and chromatin modifications (methylation, phosphorylation, acetylation), regulation of gene expression by non-coding RNAs, genome instability and any other force that modifies animal phenotype. Methylation of DNA is part of epigenetics that describes the heritable changes to DNA and chromatin that are passed on to daughter cells (Parle-McDermott & Ozaki, 2011). Methylation of DNA in the region of a gene is generally associated with gene expression being switched off, or DNA not methylated associated with a switched-on gene expression.

Nutrigenomics refers to the interaction between the nutritional environment and gene expression (Cassar-Malek *et al.*, 2008). Dietary nutrients can influence the gene expression directly or indirectly, and therefore it can affect transcription (transcriptomics), protein expression (proteomics), metabolic and/or signalling status of cells (metabolomics) and as an end result, tissues, organs and the entire organism. Fenech (2008) defines nutrigenomics as the way genetic variation responds to nutrition that includes both micro- and macronutrients. In Table 2.1, a summary of studies show the phenotypic effects observed from selected dietary factors on DNA methylation.

Table 2.1 Effects of selected dietary factors on DNA methylation in animals.

Nutrient/diet component	Observation relevant to DNA methylation	References
Methyl donor	Maternal supplementation with methyl donors reversed the effects of maternal bispehnol-A exposure during pregnancy	Dolinoy *et al.*, 2007
Folic acid	Changes in tp53 gene expression and DNA methylation status of intrauterine growth retarded rats were reversed by dietary folic acid supplementation	He *et al.*, 2015
Protein	Dietary supplementation with folic acid prevented hypermethylation in imprinting control reagion of IGF2 and H19 genes with low protein diet only	Kovacheva *et al.*, 2007
Protein	Promoters of 204 genes were differentially methylated in murine foetal liver in response to low-protein feeding during pregnancy	Van Straten *et al.*, 2010
Fatty acids	Fat exposure during development induces persistant changes in hepatic PUFA status in offspring through epigenetic regulation of fatty acid desaturase gene (Fads2) transcription	Mennitti *et al.*, 2015

Nutrigenomics is of particular interest to the management of livestock animals for production purposes and can be used to provide a basis for understanding the biological activity of dietary components (Li, 2015). The goal of nutrigenomics is to maximize health and production by feeding the animals according to the genetic potential or requirements (Seamans & Cashman, 2011). The fact that nutritional components can interact with the genome, especially through transcription regulators, opens the possibility for fine-tuning the biology of the organism through refined manipulation of the diet (Bionaz *et al.*, 2015). Nutrigenomics may contribute in designing new nutritional and management strategies for controlling metabolic processes in livestock by managing the gene expression rather than the traditional animal performance or nutritional responses (Dawson & Harrison, 2007).

Nutrigenomics encompasses the nutrient-gene interactions through intermediate action of transcriptional regulatory factors in the short to medium term and epigenetic factors in the medium to long term (Bionaz *et al.*, 2015). Transcription factors (TF) can be activated or repressed directly or indirectly by bioactive nutrigenomic molecules. Thus, the interaction between the gene and the nutrient is TF-mediated interaction with the genome rather than a direct binding of nutrients to the genome. Nutrigenomics can be divided into transcriptomics, proteomics and metabolomics (Mariman, 2006; Zdunczyk & Pareek, 2009). These technologies, known as the ‘omics’ technologies,

should result in a better understanding of the molecular processes in animal organisms and a more accurate evaluation of the biological properties of feeds. Transcriptomics has been explained in the previous section. Proteomics is the study of all the proteins in a specific cell, tissue or compartment at a specific point in time (Loor *et al.*, 2015). A range of alternative RNA processing and post-translational modifications enable a gene to produce a number of proteins differing in physiochemical and functional characteristics (Mariman, 2006). Furthermore, mRNA and protein concentration does not always change in unison; cells are able to respond to a changed environment through enzymatic modifications of proteins. In livestock studies, the research is usually focussed on the assessment of the influence of nutrient components on the proteome of a selected organ (Zdunczyk & Pareek, 2009). The proteome is the protein equivalent of the genome and is determined by the sequence, type and number of its nucleotides. Metabolomics is the sum of all the metabolites in a biological system: organism, organ, tissue or cell (Müller & Kersten, 2003). It excludes DNA, RNA and protein. Metabolomics may be suited to assess exposure to nutrients for measuring compliancy during a dietary interaction or for determining the bioavailability of nutrients (Mariman, 2006). It offers a platform for comparative metabolite analysis between specific nutritional treatments that reflect the dynamic processes underlying cellular process (Loor *et al.*, 2015). The metagenome (metabolic potential) and its expressed genes (metatranscriptome) can be used to model microbiome functions.

2.5 Methods of measuring gene expression

Over several decades, techniques have been improved for studying gene expression in mammals with next generation sequencing (NGS) being the most recent and cost-effective method for studying gene expression (Buermans & den Dunnen, 2014). Many processes can be studied using sequencing, e.g. replication, transcription (transcriptome), translation (proteome), methylation and nuclear DNA folding. Table 2.2 provides a summary of the development of transcriptome technology. Northern blot technique was one of the first techniques used to study gene expression (Alwine *et al.*, 1977) but it had a low-throughput, used radioactivity and required large amounts RNA (Morozova *et al.*, 2009). This method of RNA detection involved the separation of cellular RNA by size using gel electrophoresis that was transferred to a solid support (e.g. nylon or microcellulose membrane) where the presence or abundance of the RNA of interest was measured by the hybridization of a complementary radioactively labelled nucleic acid probe (Doğan *et al.*, 2015). A major disadvantage was the complexity and time- consuming nature and at the same time requiring large amounts of RNA.

Table 2.2 A summary of the development of transcriptome technologies (Adapted from Morozova *et al.*, 2009)

Year	Development
1965	The first RNA molecule was sequenced
1977	Northern blot technique and Sanger sequencing method developed
1989	RT-PCR experiments for transcriptome analysis
1991	The first high-throughput EST sequencing study
1992	Differential display for the discovery of differentially expressed gene was introduced
1995	The development of microarray and serial analysis of gene expression (SAGE) methods
2001	Draft of human genome completed
2005	First next-generation sequencing technology introduced (454 Life Sciences)
2006	The first use of next-generation sequencing technology for transcriptome sequencing.
2009	Bovine genome sequenced (Elsik *et al.*, 2009)
2010	The first RNA-seq study done in livestock (Medrano *et al.*, 2010)
2010/2011	Release of Ion Torrent Personal Genome machine, Pacific Biosciences (PacBio) RS and Illumina MiSeq (van Dijk *et al.*, 2014; Quail *et al.*, 2012)
2014	Illumina releases NextSeq 500 and HiSeq X Ten (Buermans & den Dunnen, 2014)

Reverse transcriptase PCR is a method used for the detection of mRNAs and their quantification (Bustin, 2000). In this technique the mRNA is reverse transcribed into complementary DNA and amplified using PCR primers that is specific for the gene of interest. Quantitative RT-PCR protocols measure the mRNA abundance by monitoring the accumulation of the amplification product and takes advantage of PCR using it in the reverse transcription method to amplify transcripts (Doğan *et al.*, 2015). The RT-PCR method is based on a specific or non-specific detection chemistry that allows the quantification of the amplified product that overcame the disadvantages of the Northern blotting technique and resulted in more efficient analysis of gene expression (Becker-Andre & Hahlbrock, 1989). It is flexible, cost-effective and not time-consuming. The primary limitation of QT-PCR is that it only focusses on one gene at a time; it is not an independent transcriptome profiling method with high-throughput data. This method is however being used as a validation step in many gene expression studies.

The development of microarray technologies enabled researchers to characterize the expression levels of thousands of transcripts in different cell types and compare expression levels of the genes during various physiological conditions (Schena *et al.*, 1995). Isolated mRNA is used

to synthesize fluorescently tagged cDNA followed by incubation with a chip where hybridization takes place depending on the complementarities of probes in the microarray (Doğan *et al.*, 2015). The specific gene's expression is quantified by detecting these fluorescent signals. Microarrays use high-throughput transcriptomic arrays containing 30 000 transcripts to reveal the genetic regulation in a set of biological conditions that relate to the phenotypic differences (Kadarmideen, 2014). It was observed in microarray that some cross-hybridization of probes can occur between genes that have almost identical sequences (Wickramasinghe *et al.*, 2014). The scope of the scanner limits the microarray's dynamic range (Marguerat *et al.*, 2008). Transcriptomic studies that uses microarray gene expression profiling was and remains popular in livestock studies (Kadarmideen, 2014; Wickramasinghe *et al.*, 2014). The limitations of this technique however include: the high-background noise detection due to cross-hybridization of probes, the inability to detect novel transcripts, the inability to study the coding sequence of transcripts that has already been found and an overreliance on existing knowledge of the genome sequence (Marioni *et al.*, 2008). According to Cassar-Malek *et al.* (2008) one of the limitations of microarrays is related to the multiple gene products and the difficulty in detecting subtle transcriptional changes reflecting the replacement of a protein isoform by another one, for example, such as those occurring during development (Lehnert *et al.*, 2007).

Next Generation Sequencing (NGS) of the whole-genome has provided the ability to build comprehensive maps of genetic variation that includes millions of single nucleotide variants and non-coding RNA (Koboldt *et al.*, 2013; Mutz *et al.*, 2013). Next Generation Sequencing of DNA includes the cleaving of the DNA sample genome into short reads of 50 or 100 base pairs and aligning them to the reference genome (Kadarmideen, 2014). The sequence depth is used to show how well the DNA can be mapped to the reference genome. The sequence depth can be defined as the measure of the number of reads covering a specific nucleotide position. If there is a difference between the sample genome and the reference genome of a specie, they are identified as 'genetic variant'. The transcriptomics variant of pyrosequencing technology is called short-read massively parallel sequencing or RNA-seq (Denoeud *et al.*, 2008). RNA-seq determines and counts the mRNA sequences. It estimates RNA expression levels in cells or tissues with higher accuracy compared to microarrays. The results from RNA-seq experiments can be compared directly without any normalization methods necessary. RNA-seq has been known to unravel complexities in the transcriptome such as allele-specific expression and novel promoters and isoforms, gene expression, detection of alternative splicing and RNA editing (Baldwin *et al.*, 2012). RNA-seq shows a detailed view of the transcriptome (Nagalakshmi *et al.*, 2010) by assembling millions of reads that can reveal new exons and introns, the mapping of their boundaries, the identification of new splicing variants and the monitoring of allele expression.

RNA-seq studies the complexity of the transcriptome and has provided an improved understanding of transcription regulation and associated networks (Cloonan & Grimmond, 2008;

Loor *et al.,* 2015). It provides biological insight into understanding the underlying molecular basis of metabolic differences among different tissues (Lee *et al.*, 2014). In RNA-seq, 96-100% of the sequence reads needs to be known to map to the reference genome and this enables the accurate identification of homolog and repetitive sequences transcripts that are excluded from microarray analysis. Wickramansinghe *et al.* (2014) uses a metaphor to illustrate the difference between microarray technology and RNA-seq; 'Microarray is a closed architecture platform with a predesigned chip while RNA-seq is an open architecture platform.'

In Table 2.3 the advantages and disadvantages of RNA were summarized. A significant advantage of RNA-seq is the accurate quantification at all expression levels because of the wider dynamic range leading to a comparison of gene expression among the highly expressed genes.

Table 2.3 Advantages and limitations of RNA-seq technology (Wickramasinghe *et al.*, 2014)

Advantages of RNA-seq
Decreased cost compared to technologies based on Sanger sequencing
Gene expression intensities are countable and provide absolute values
Provides accurate quantification and comparison of relative gene expression levels
New splice junctions and exons can be identified and studied
Allele specific expression can be studied and the parental allele ratios obtained
Provides SNP in expressed exons
Used to build de novo gene models in species with fewer available genomic resources
Limitations of RNA-seq
A higher degree of computational resources is required for de novo assembly of genes
During PCR amplification in RNA-seq library preparation a degree of bias can be introduced
No established methods to compare the error rates between different NGS technologies
High requirement of computer space for data storage
Relatively higher cost compared to the microarray experiment

Gene expression is estimated by counting sequencing reads with RNA-seq technology. In organisms were the reference genomes are of good quality, RNA-seq technology can be applied to obtain a large amount of useful information (Wickramansinghe *et al.*, 2014). During standard RNA-seq library preparation the double stranded cDNA is sheared and sequenced, but the directionality of the fragment can't be identified and the precise mapping of the fragment to the specific strand in the genome is lost. A major requirement for NGS techniques is the requirement of high performance computing facilities and storage for large datasets (Baldwin *et al.*, 2012; Wickramansinghe *et al.*, 2014). The general workflow from RNA-seq for gene expression are shown in figure 2.2 below.

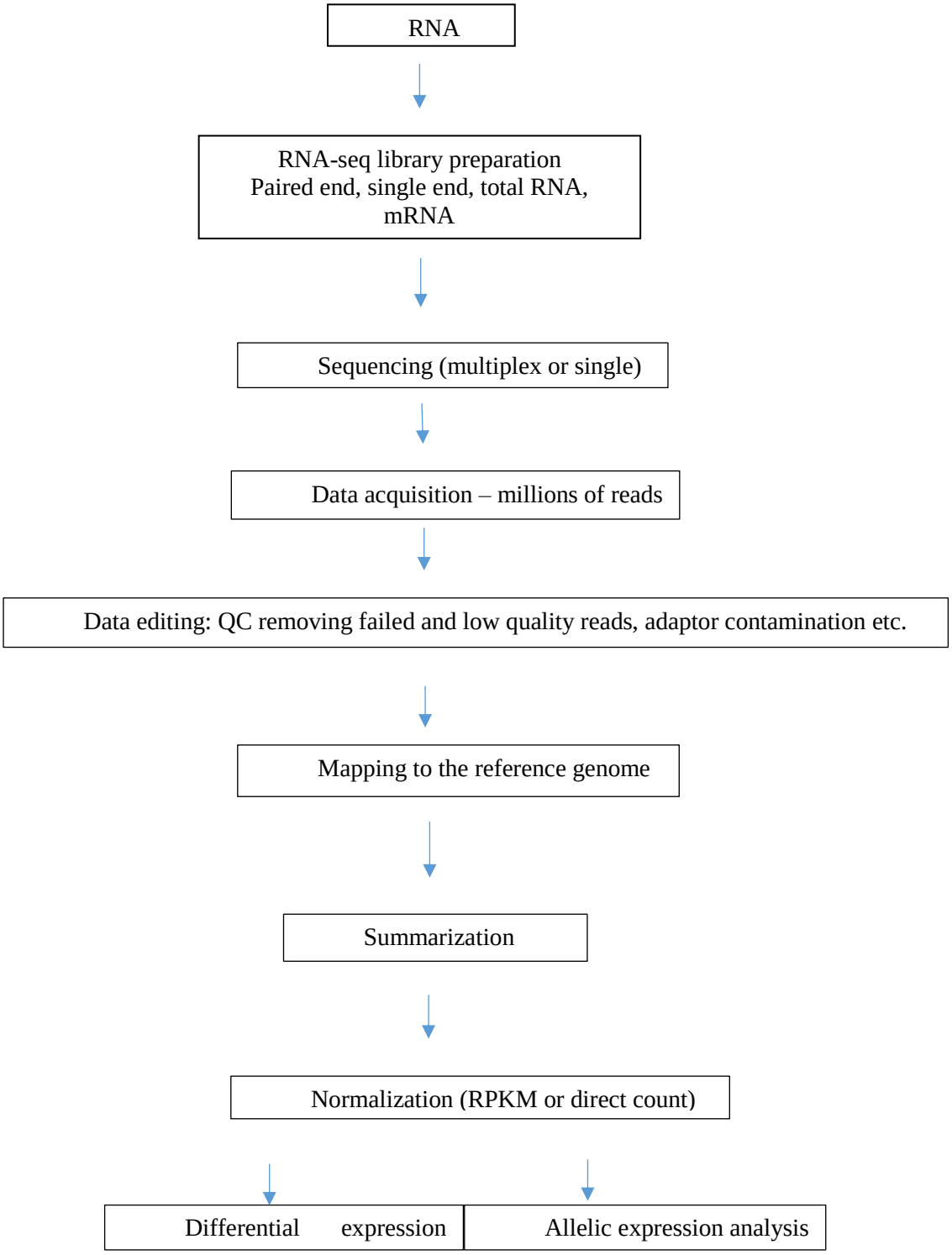

Figure 2.2 The general workflow of RNA-seq for gene expression analysis including all the important steps using any of the platforms for RNA-sequencing (Adapted from Wickramasinghe *et al.*, 2014).

The primary data that results from RNA-seq technology is image-related files that take up a large amount of computer storage space (Mutz *et al.*, 2013). These raw image files must be converted into short sequence reads. The raw data files are fluorescence signals which are

converted into base sequences by algorithms provided by the manufacturer. These reads are then aligned to the reference genome or transcriptome, followed by counting the number of reads and the gene expression level is then calculated by the peak using algorithms. Statistical tests are used to determine the differential gene expression. The cost of this technology is becoming relatively more affordable (Wickramasinghe *et al.*, 2014), but to examine the expression of less abundant transcripts, higher coverage of the transcriptome is needed. This increase in sequencing depth remains expensive.

2.5.1 Commercial Platforms for RNA-seq

RNA-seq is rapidly emerging as the major quantitative transcriptome profiling system (Wickramasinghe *et al.*, 2014; Liu et al., 2012). Different companies developed different sequencing platforms all based on pyrosequencing variations (Mutz *et al.*, 2013) (Figure 2.3). All of these sequencing platforms use short fragments called reads to investigate genome sequences. The two most common variations based on pyrosequencing principles are Roche/454 and the Pyromark ID system (Pareek *et al.*, 2011).

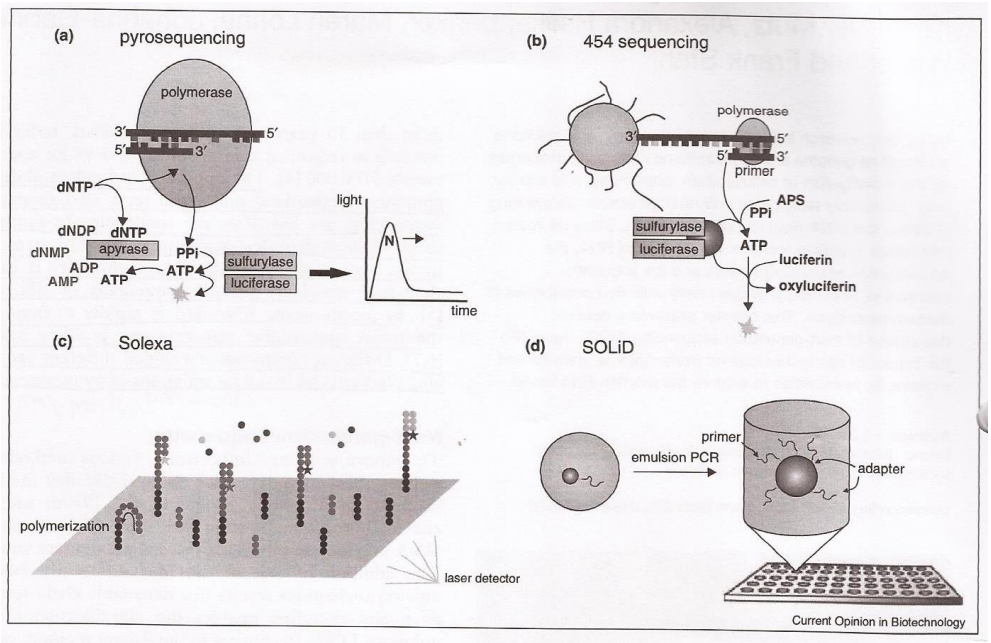

Figure 2.3 The different technologies used for RNA sequencing (Mutz *et al.*, 2013).

The second developmental stage of pyrosequencing was the invention of surface-based systems. These systems also follow the principle to detect every step that incorporates single nucleotides (Buermans & den Dunnen, 2014). This provides options for high-throughput functional transcriptomics. Discovery of transcription factor bindings or non-coding RNA expression profiling can be established using these methods. The Genome analyser and SOLiD (Sequencing by Oligo Ligation Detection) are two most common variations (Liu *et al.*, 2012). A comparison of the

sequencing platforms described in Figure 2.3 is detailed in Table 2.4 or can be found in Pareek *et al.* (2011).

Table 2.4 RNA sequencing platforms comparison (Wickramasinghe *et al.*, 2014)

Platform	Method of amplification	Sequencing chemistry	Instruments	Maximum read length	Reads per run
Roche/454	Emulsion PCR on agarose beads	Pyrosequencing	GS FLX+	~ 1000bp	1 000 000
			GS Junior	~ 400 bp	70 000
Illumina	Bridge amplification as clusters on the flow cell surface	Sequencing by synthesis	HiSeq 2500/1500	~150 bp	300 millions – 3 billions single reads
			HiSeq 2000/1000	~150 bp	300 millions – 3 billions single reads
			GA IIx	~150 bp	320 million single reads
			MiSeq	~300 bp	~17 million single reads
SOLiD	Emulsion PCR on beads catalysed by DNA ligase	Oligo ligation and detection	5500 system	~75p	100 million paired-end reads
			5500xl system	~75 bp	100 million paired-end reads
Helicos	No amplification directly measures the single nucleotide molecules	Sequencing by synthesis and single molecule imaging	HeliScope single molecule sequencer	~55 bp	420-700 Mb per channel

Pacific Biosciences (PacBio) RS utilizes single molecule real time (SMRT) sequencing. DNA polymerase molecules are attached to the bottom of 50 nm-well termed zero-mode waveguides (ZMWs) (Eid *et al.*, 2009). The polymerase carries out a second strand DNA synthesis with fluorescently labelled γ-phosphate nucleotides. The fluorophores, attached to the nucleotides located in the area of the polymerase at the bottom of the well, are excited by the energy that penetrates the short distance. A distinctive pulse of fluorescence is released as each base is incorporated and the fluorescence pulse is detected in real time. Sequencing runs last minutes or hours rather than days as in other platforms (van Dijk *et al.*, 2014). PacBio creates long reads that are useful for *de novo* assemblies (Quail *et al.*, 2012). A disadvantage of PacBio is that the DNA-input requirements can be prohibitory.

Ion Torrent Personal Genome Machine (PGM) is one of the more recent sequencing technologies launched in 2011 (Quail *et al.*, 2012). A direct connection between the chemical and the digital information is created with this technology, enabling simple, fast massively scalable sequencing (Pareek *et al.*, 2011). It uses the power of semi-conductor technology to detect the protons released as nucleotides incorporated during synthesis (Rothberg *et al.*, 2011). DNA fragments with specific adapter sequences are linked to and clonally amplified by emulsion PCR on the surface of Ion Sphere Particles that are 3-micron diameter beads. These beads are loaded into proton sensing wells on a silicon wafer and sequencing is primed from a specific location in the adapter sequence. Each of the four nucleotides are introduced sequentially as sequencing proceeds and the protons are released with the detection of a signal in proportion to the number of bases incorporated. PGM does not rely on the optical detection of incorporated nucleotides using fluorescence and camera scanning (van Dijk *et al.*, 2014). This non-reliance resulted in higher speed, smaller instrument size and lower cost. The disadvantage of Ion Torrent is that its semiconductor sequencing is not recommended for sequencing of genomes extremely rich in AT (Quail *et al.*, 2012). This is due to the severe coverage bias observed which is likely introduced during amplification.

2.6 Software programs for RNA-seq analysis

RNA-seq offers the opportunity to discover new genes and transcripts and to measure transcript assays (Mortazavi *et al.*, 2008; Nagalakshmi *et al.*, 2010). The output that is generated from these software programs is gene or transcript abundances. Gene abundance is the number of reads produced from each gene and can be used to detect which genes are expressed at significantly different levels (Pertea *et al.*, 2016). Data from these experiments can detect genes and gene variants that are not included in standard annotation and to discover conditions in which distinct isoforms of a single gene are differentially regulated and expressed. However, these experiments generate enormous volumes of raw sequencing reads. In 2012, instruments generated more than 500 gigabases in a single run (Trapnell *et al.*, 2012). Data generated form RNA-seq experiments

must be analysed with efficient and statistically principled algorithms. Efficient and accurate quantification of RNA-seq transcript abundances is a problem due to the wide range of technical biases that affect the RNA-seq fragmentation, amplification and sequencing process (Roberts *et al.*, 2011; Love *et al.*, 2014). Analysis tools for RNA-seq data can be classified into three categories. Firstly, those that are used to align the reads. Secondly, the software tools that are used for transcript assembly or genome annotation and lastly, those that are used for transcript and gene quantification. Most of these software programs require multiple CPU cores to be able to process the RNA-seq data.

One possible pipeline that can be used is that of Trapnell *et al.* (2012). In this pipeline, the Tuxedo package is used that incorporates BowTie (Langmead *et al.*, 2009), TopHat (Trapnell *et al.*, 2009) and Cufflinks (Trapnell *et al.,* 2010). TopHat as the program that aligns reads to the genome and discovers transcript splice sites. TopHat uses BowTie as an alignment program. Assembling the reads into transcripts is the role of Cufflinks. Part of the Cufflinks package is Cuffdiff, which uses the aligned reads from the treatments and through strict statistical analysis determines the genes that are differentially expressed. CummeRbund uses the output from Cuffdiff to transfer the data in easy-to-read figures and charts. However, these programs do not address all RNA-seq applications. One of the major limitations of these programs is that a reference genome (sequenced genome) is required. The protocol explained in Trapnell *et al.* (2012) also assumes that SOLiD or Illumina sequenced machines are used. The analysis from data generated by 454 or the capillary electrophoresis approach is substantially different from the approach used in this pipeline.

Another pipeline that can be used is described by Pertea *et al.* (2016) where HISAT (Kim *et al.*, 2015), StringTie (Pertea *et al.*, 2015) and Ballgown (Frazee *et al.*, 2015) are applied to analyse the data. HISAT align the reads to a genome and discovers transcript splice sites. In comparison to TopHat, HISAT runs faster and requires less computer memory. StringTie assembles the alignments into transcripts, creating multiple isoforms as necessary and estimating the expression levels of all genes and transcripts. Ballgown takes the output (transcripts and expression levels) from StringTie to determine differentially expressed genes between treatment groups. As Ballgown is part of the R/Bioconductor package it assists in visualizing the results. In this protocol, it is optional to provide an annotation file, but recommended. This protocol can handle data from many RNA-seq experiments, however there is data that it will not be able to accurately analyse. This includes data that requires pre-processing to remove contaminants, adapters and low quality sequences from the raw data. There are programs such as Trimmomatic (Bolger *et al.*, 2014) and FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) that can be used to ensure high quality data. One of the other limitations of this protocol is that it assumes Illumina was the sequencing machine that was used to generate the data. Longer reads from third generation sequencers (Pacific Biosciences) may require different software. StringTie uses GTF files, while most other programs use GFF (gene finding format). GFF annotation files will need to be converted to GTF files.

Salmon (Patro *et al.*, 2017) uses a dual-phase parallel inference algorithm and feature rich bias models with an ultra-fast read mapping procedure for quantifying transcript abundance from RNA-seq reads. Software programs that use alignment of sequencing reads to the genome or transcriptome require substantial computational resources and don't scale well with the rate at which the data is produced (Kodama *et al.*, 2012). Salmon accounts for sequence specific, fragment GC-content and potential biases. This program consists of three phases; a lightweight mapping model, a phase that estimates initial expression levels and model parameter online and an offline phase that refines the expression estimates. As Salmon does not visualize the results, it is important to make use of the Bioconductor package that processes the data using R (Gentleman *et al.,* 2004). Along with the Bioconductor packages, the DEGseq (Wang *et al.*, 2010) or DESeq2 (Love et al., 2014) can also be used.

There are many other software programs that can be used to analyse RNA-seq data (Patro *et al.*, 2014; Kim *et al.*, 2013; Li *et al.*, 2011; Grabherr *et al.*, 2011; Schultz *et al.*, 2012). The programs and pipelines mentioned above is those that are mostly used in the RNA-seq bioinformatic community.

2.7 Applications of gene expression studies in livestock

A number of gene expression studies in livestock have focused on meat and dairy traits. These studies focused mainly on the expression of the marbling genes (Chen *et al.*, 2015; Lee *et al.*, 2007; Sasaki et al., 2005), the expression of genes in the mammary gland (Capuco *et al.*, 2011; Huang *et al*., 2012; Wickramasinghe *et al.*, 2012) and in reproduction (Mamo *et al.,* 2012; Walsh *et al.*, 2012; Driver *et al.*, 2012; Chitwood *et al.*, 2013). The first RNA-seq study in cattle was done by Medrano *et al.* (2010) in a comparative transcriptome analysis of milk somatic cells and mammary tissue. The study demonstrated the robustness of RNA-seq analysis of animal tissues on a global scale. The analysis of transcriptomic results can be used to compare the expression levels of genes in different tissues involved in complex metabolic pathways (Wickramasinghe *et al.*, 2014). There are a number of studies that explored large-scale transcriptomic adaptations in response to the plane of nutrition (normal feeding vs. underfeeding) in the skeletal muscle of cattle (Byrne *et al.*, 2005; Lehnert *et al.*, 2006; Reverter *et al.*, 2003). Some of these studies will be explained below.

Gene expression based research has focussed on the identification of molecular processes involved in meat quality traits, e.g. toughness and marbling (Lehnert *et al.*, 2006). The measurement of mRNA for multiple genes and their networks in pathways should expand the knowledge of muscle and fat development in response to nutrition, genotype and their interaction (Lehnert *et al.*, 2006) due to regulations of metabolic enzymes in mammals at the transcriptional level (Desvergne *et al.*, 2006). Other studies also focussed on the muscle growth potential (Cassar-Malek *et al.*, 2008; Sudre *et al.*, 2004) and the effects of nutritional changes (Byrne *et al.*, 2005), all of them influenced the

composition of muscle tissue. Intramuscular fat, which influences the quality of the meat, has also been examined (Wang *et al.*, 2005; Lee *et al.*, 2007). In the study by Sadkowski *et al.* (2014), RNA-seq was used to compare the number of upregulated and downregulated marbling related genes between three breeds to identify the putative candidate IMF-related genes in the semitendinosus muscle. Sadkowski *et al.* (2014) found that there were nine validated marbling-related genes that were downregulated or upregulated. Damon *et al.* (2012) observed that there was a significant difference between the gene expressions of two pig breeds, Large White breed commonly used in pig production and Basque breed indigenous to France and concluded that energy metabolism and fat deposition differences observed in the gene expression were the result of breed differences. Bongiorni *et al.* (2016) also observed differences between breeds in a study on the *Longissimus dorsi* of Italian Maremmana and Chianina cattle breeds. The study focussed on the tenderness of meat and concluded that the difference in gene expression between the breeds was due to different selection goals. Selection can therefore change the gene expression of the animals. A study done by Teixeira *et al.* (2017) observed gene expression differences between the *longissimus thoracis* muscle of Angus and Nellore cattle breeds.The main difference in gene expression between these two breeds was with regard to intramuscular fat deposition. This gene expression difference may be the result of subspecies differences as Angus is a Bos taurus and Nellore is a Bos indicus breed type. The difference in gene expression between breeds was also seen in sheep (Miao *et al.*, 2015). Through these studies, it can be concluded that gene expression can change through a variety of factors. Factors like the management of the herd, the nutrition fed, the breed itself and the current physiological phase of the animal, can change gene expression.

Gene expression studies done on dairy cattle focussed on the gene expression in the mammary gland which depends on the physiological condition of the cow. Wickramasinghe *et al.* (2012) studied the expression of a cow's genes throughout lactation and found that at peak lactation milk somatic cells had the highest number of expressed genes and at transition the milk somatic cells had the lowest number of expressed genes. This can lead to the conclusion that the physiological state of an animal may have an influence on the expression of certain genes. Peak lactation had the highest expression of genes with antioxidant activity; while in late lactation genes that were highly expressed was genes with immune activity. They finally concluded that the milk somatic cells have the ability to adapt to different molecular functions according to the biological need of the animal. Gigli & Maizon (2015) also found that management factors like milking frequency, mastitis, photoperiod and heat stress can effect gene expression. Changes in milking frequency and the presence of mastitis were associated with chromatin modifications. The endurance of photoperiod and heat stress over time suggested an epigenetic mechanism. A better understanding of the epigenetic responses of the mammary gland can lead to the development of new management procedures which could result in higher quality milk products and a better consideration for the animal's welfare. Cattle genetics and gene expression might be an important factor to consider when

developing management strategies to manipulate skeletal muscle composition (Graugnard *et al.*, 2009).

There are four focus areas in nutritional studies where gene expression profiling of the skeletal muscle can impact livestock production, namely (1) animal production (especially breeding animals' longevity), (2) improving the efficiency of production, (3) the quality of the products and (4) the health of the animals (Reecy *et al.*, 2006). The early nutrition of the calf and the nutrition of the mother while carrying the calf has an influence on the performance of the calf later in its life (longevity of the animal). Gutiérrez *et al.* (2014) found that the herbage allowance of the mother during gestation and lactation has an effect on the calf body weight, body composition and calf *Semitendinosus* muscle fiber diameter and expression of IGF-I system and adipogenesis related genes. They found that at birth the muscle peroxisome proliferator activated-receptor γ (PPARG) mRNA was greater in animals that had a high amount of herbage allowance than the animals that had a low amount of herbage allowance. This could mean that the calves born from mothers that had a high roughage allowance have a greater potential for marbling due to higher expression of marbling genes. This shows that even a small change in the management of the previous generation can influence gene expression.

It is known that gene expression can also be influenced by the feed ingredients in the diet of the animals. Baldwin *et al.*, (2012) performed a study on Angus steers to investigate the result of different propionate levels on the *Longissimus lumborum* transcriptome. They found that steers who received propionate had a higher weight gain over the period of the trail. Total daily Nitrogen (N) intake did not differ between the treatment and the control, however faecal N excretion was reduced and this increased the whole body N retention. The study found through RNA sequencing that the steers given the propionate infusion had less genes transcribed while the control animals, who did not receive the propionate infusion, had more transcribed genes. The majority of the genes expressed were muscle contraction-related, such as various myosins, tropomyosins and myosin-binding proteins and glycogen phosporylase (muscle form). The changes observed in the *Longissimus Lumborum* transcriptome appear to be reflective of the changes in lipid metabolism within this muscle, presumably in support of increasing marbling and other growth-specific and metabolic control points.

Another example of the effect of feed ingredients is a study done on the effect of flax meal and flax oil supplementation on the mammary tissue's gene expression (Lima *et al.*, 2015). They found that when flax oil was infused abomasally there was a reduction on glutathione peroxidase (GPX) activity in the plasma and a reduction in catalase (CAT) activity in the mammary tissue. This suggests that flax oil infusion in the abomasum prevented the flax meal induced expression of GPX1 transcript. This study indicated that nutrition also has an influence on the expression of the genes in the mammary gland. Mach *et al.* (2011) found that 972 genes were differentially expressed in the mammary gland when the grazing dairy cows were supplemented with unsaturated fatty acids (UFA)

(rapeseed oil, soybean oil or linseed oil). This suggests that there is a large degree of transcriptomic adaptation to the dietary UFAs.

It has been shown that the level of the starch or protein in the diet resulted in different expressions of the genes. Graugnard *et al.*, (2009) studied the effect of low starch diets and high starch diets on Angus and Angus x Simmental cattle. High-starch/low-fiber diets provide readily-available sources of energy for growing muscle in early-weaning management systems by shifting ruminal fermentation towards higher propionate production (Myers *et al.*, 1999). The authors found that the mRNA of INSIG1 had greater upregulation in all their groups except the crossbred steers fed the high-starch diet. The higher expression of an insulin gene provides a feedback signalling mechanism to restrict both lipogenesis and adipogenesis. The peroxisome proliferator activated receptor coactivator-1α (PPARGC1A) and -1β (PPARGC1B) are also involved in simulating oxidative metabolism in skeletal muscle. In their study they observed that only Angus steers fed the low starch diet had a significant increase in transcript abundance of PPARG. PPARGC1B was up-regulated in steers fed the high starch diet. They also found a positive correlation between PPARGC1A and PPARGC1B with acyl-CoA synthetase (ACSL1), encoding a protein showing to cannel fatty acids towards oxidation in muscle tissue. Gene expression patterns suggest that dietary starch level might alter pathways associated with intramuscular adipose tissue development.

2.8 Candidate genes and physiological pathways

Gene expression analysis provides essential information on the upregulation or downregulation in different physiological pathways. The physiology of genes should be known to be able to understand the chain reaction that causes the gene to be upregulated or downregulated. The physiological pathways of a few genes will now be described that were up- or downregulated in various studies in gene expression in bovine production. In Table 2.5 there is a summary of some studies done on the gene expression of certain and relevant genes in the bovine that can possibly be upregulated or downregulated in energy metabolism. The gene expression analysis profile of these genes could be of great interest with regard to muscle development and beef quality. The physiology of these genes is described below the table.

Table 2.5 Studies done on gene expression in bovine.

Trait	Genes	Method	Reference
Lipid browning	WDR3, HDAC3, HPRT1	qPCR	Cao *et al.*, 2017
Marbling	MyoD, CLCN, COL, MyoG	RNA-seq	Chen *et al.*, 2015
Fat metabolism	LPL, DGAT1, PPARG, LEP	qPCR	Dervishi *et al.*, 2011
Fat deposition	HSL, CPT, FASN, LPL, ACOT	qPCR	Fang *et al.*, 2017
Energy metabolism	CDIPT	qPCR	Fu *et al.*, 2014
Meat quality	GAPDH, HPRT, TBP	qPCR	Giusti *et al.*, 2013
Adipogenesis	PPARG, THRSP, SCD	qPCR	Graugnard *et al.*, 2009
Adipogenesis	GHR, IGF, PPARG, SREBF1	qPCR	Gutiérrez *et al.*, 2014
Muscle development	MEF2C	qPCR	Juszczuk-Kubiak *et al.*, 2014
Muscle development	FSTL, IGFBP, GDF	Microarray	Lehnert *et al.*, 2007
Meat quality	CAST, LEP, DGAT	qPCR	Niciura *et al.*, 2012
Marbling	ATP6, TPI1, TNNT1, MDH2	qPCR	Shin & Chung, 2016
Marbling	CDC10	qPCR	Tong *et al.*, 2015
Fat deposition	ACTA, FABP, ANKRD,THRSP	Microarray	Wang *et al.*, 2005
Residual Feed Intake	NPY, GSK, PEPCK, IRS	Microarray	Xi *et al.*, 2015

The role of the MEF2 proteins in muscle development is to control myogenesis and morphogenesis by cooperating with the myogenic basic helix-loop-helix transcription factors (e.g. MyoD, myogenin) (Black & Olson, 1998), homeobox proteins (e.g. tinman, Gax) (Cripps *et al.*, 1998) and/or GATA factors (Morin *et al.*, 2000). MEF2 is the myocyte enhancer factor 2 and is part of the family of MADS (MCMI-agamous-deficient-serum response factor). This is a family of transcription factors that is highly expressed in cells of muscle lineage. They are important regulators of gene expression during skeletal, cardiac and smooth muscle development (Black & Olson, 1998). MEF2 proteins also have an additional level of control by regulating gene expression. MEF2 proteins post-transcriptionally repress gene expression by binding to the 3' UTR of the mRNA target and disrupting mRNA translation and stability (Zhao *et al.*, 2011). Despite extensive studies on MEF2 during embryogenesis, little research has been done during postnatal growth in the skeletal muscles (Juszczuk-Kubiak *et al.*, 2014). One of the MEF2 proteins ME2C also upregulates peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α in skeletal muscles (Sevane *et al.*, 2013). It has also been shown that MEF2C together with the nuclear factor of the activated T cells (NFAT), regulates the activity of myoglobin promoter and troponin 1 enhancer (Chin *et al.*, 1998). This suggests that there may be a relationship between MEF2C expression and meat quality. The bovine MEF2C gene is made up of eleven exons coding for 367 amino acid proteins (Wu *et al.*, 2011) and

has been mapped to chromosome 7. This chromosome contains quantitative trait loci's (QTL) responsible for average daily gain, body and carcass weight (Casas *et al.*, 2003) and fat thickness in the *longissimus dorsi* muscle (Ferraz *et al.*, 2009). Both the biological significance and the chromosome localization suggests that the bovine MEF2C gene could be a promising functional and positional candidate gene responsible for carcass and meat quality traits in cattle (Juszczuk-Kubiak *et al.*, 2014).

Another family of genes that might be influenced by dietary factors are the MRF (muscle regulatory factors) gene family (Houba & te Pas, 2004). Four structurally related transcription factors, namely myogenin, MyoD1, MRF4 and myf-5 belong to this family. They modurate both skeletal muscle fibre development and hypertrophic postnatal growth. The MRF family activates skeletal muscle differentiation stage-specific genes and therefore may be important for the amount of meat deposited in animals.

Peroxisome proliferator activated receptor γ (PPARG) is a critical transcriptional regulator of genes controlling energy metabolism, adipogenesis and maintenance of a differentiated state (Rosen & MacDougald, 2006). The tissue where it is expressed determine the function of the gene regarding energy metabolism; whereas PPARG has a lipogenic and adipogenic effect in adipocytes and hepatocytes (Kersten, 2001). It promotes fatty acid oxidation in the muscle, which leads to a decrease in lipid availability (Lapsys *et al.*, 2000). PPARG activation in the muscle through genes like SIRT1 and PPARGC1A promotes the expression of lipoprotein lipase and leads to increased fatty acid oxidation, glucose uptake and mitochondrial biogenesis when the glucose level are low. PPARGC1A has a key function in activating a variety of nuclear hormone receptors and transcription factors regulating energy metabolism (Puigserver & Spiegelman, 2003). PPARG over-expression has a direct influence on SCD and DGAT1 activity when glucose intake is increased (Sevane *et al.*, 2013). Adipocyte differentiation is induced by the up-regulation of PPARG in vitro. No other factor has been discovered that promotes adipogenesis in the absence of PPARG (Rosen & MacDougald, 2006). Terminal differentiation of adipocytes requires up-regulation of mRNA of fatty acid binding protein 4 (FABP4), G6PD, FASN and ACACA, which are under the control of PPARG (Hausman *et al.*, 2009).

Forkhead box 01 (FOX01) belongs to a protein subfamily that influences a variety of cellular functions, including energy metabolism through the regulation of master transcription factors like PPARG (Kousteni, 2012). FOX01 transcription factors also regulate the expression of myostatin (GDF8) and contribute to the control of muscle cell growth and differentiation (Allen & Unterman, 2007).

CDP-diacylglycerol-inositol 3-phosphatidyltransferase (CDIPT) is found on the cytoplasmic side of the Golgi apparatus and the endoplasmic reticulum performs the last step in the de novo biosynthesis of phosphatidylinositol (PtdIns). This is done by catalysing the condensation of cytidine diphosphate-diacylglycerol and myo-inositol to produce PtdIns and cytidine monophosphate (CMP)

(Antonsson, 1997). PtdIns metabolism and biosynthesis are of considerable interest due to its phosphorylated derivatives in energy metabolism, fatty acid metabolic pathway and intracellular signal transduction in eukaryotic cells (Ansell & Hawthorne, 1973). Breakdown products of PrdIns are second messengers that function downstream of many receptors and tyrosine kinases regulating cell growth, protein kinase C activity and calcium metabolism (Noh *et al.*, 1995). Over expression of CDIPT can enhance growth and G1 progression in NIH3T3 cells (Deguchi *et al.*, 2002) and this could lead to an overproduction of protein activity and further enhance PtdIns activity (Lykidis *et al.*, 1997). The bovine CDIPT gene is located at chromosome 25 and encodes 213 amino acids in cattle (Zimin *et al.*, 2009).

Marbling is a complex trait with various genes that influence the intra-muscular fat deposition. The diacylglycerol acyltransferase (DGAT1) gene encodes an enzyme acyl-CoA: diacylglycerol acyltransferase 1 that plays a role in the synthesis of triglycerides and its polymorphism was associated with marbling (Thaller *et al.*, 2003). It catalyses the reaction of diacylglycerol and fatty acyl-CoA to triglycerides. The leptin hormone produced by the LEP gene, influences fat deposition, daily weight gain and marbling because it regulates food intake, energy expenditure and body weight homeostasis (Kononoff *et al.*, 2005). It acts on receptors in the central nervous system to inhibit food intake and promote energy expenditure via AMPK stimulation in skeletal muscles. The role of leptinin the regulation of metabolism is observed through its action on the hypothalamic-pituitary-adrenal axis and this suggests that this gene plays a crucial role in growth (Delavaud *et al.*, 2002). Kononoff *et al.* (2005) found that a mutation in the leptin gene leads to a higher level of leptin mRNA in adipose tissue and is therefore associated with higher fat deposition. Niemann *et al.* (2011) also suggested the NPY, Agrp, CART, POMC and MCH genes for the encoding of feed intake. The FABP4 gene codes for the fatty acid binding protein. It supplies long-chain fatty acids as an important energy source for muscle growth and maintenance, forcing long chain fatty acids towards fat storage within muscle fibres (Brandstetter *et al.*, 2002). SREBF1 is a pro-adipogenic factor regulating transcriptional cascades. The transcriptional factor binds specific DNA domains eliciting transcription of genes involved in lipid and cholesterol metabolism. Changes in SREBF1 expression exert some level of control over intramuscular lipogenesis in growing cattle (Graugnard *et al.*, 2009).

There are several other genes involved in fat metabolism and myogenesis, not only the genes discussed above. The genes above where used or observed in studies related to gene expression in the bovine and therefore used as examples of genes that will be studied.

2.9 Conclusion

Gene expression is one of the most interesting fields of study but it is highly complex, involving both genetics and other factors such as nutrition and physiology. Small changes can lead to a large effect in the gene expression with significant effects on animal production and the advance in

technology makes gene expression studies more possible. From the literature studied it can be concluded that RNA-seq can quantify more genes compared to the other methods and is therefore most appropriate for gene expression studies. Even though it is computationally demanding, the data that can be gained from using RNA-seq is of high value.

There are many pathways in which genes can be regulated. However, most of the gene regulation occurs before or just after transcription. Transcription is therefore one of the most important pathways when it comes to gene expression. Thus, mRNA is one of the most important molecules to investigate with regards to gene expression.

There are many fields of animal science where gene expression is used or can be used. There are, however, more studies on the gene expression of the mammary gland and the application thereof in the dairy industry. The majority of gene expression studies done in beef cattle are on the meat quality and the nutritional factors that can influence these qualities. There is however room for expanding the applications of gene expression.

Chapter 3: Materials and Methods

3.1 Introduction

This project was divided into three phases. In phase 1 the focus was on investigating the differences between the two diets while in phase 2 the bulls were fed in a feedlot and the performance data was recorded. Muscle samples were collected from phase 2 for transcriptome sequencing in phase 3. Ethical approval was received from both the Animal Ethical Committee of the Agricultural Research Council and the Animal Ethical Committee of the University of Pretoria (Eco90-15).

3.2 The origin of the bulls

The bulls in this study were sourced from two experimental farms in the Northern Cape and Gauteng provinces, respectively. The Vaalharts Research Station is under the management of the Northern Cape Department of Agriculture, Land Reform and Rural Development and is located near Jan Kempdorp in the Northern Cape province of South Africa. The Northern Cape is the driest part of South Africa and has sweet veld grazing. Jan Kempdorp has average annual summer rainfall of 314 mm with annual summer and winter maximum temperatures ranging from 28°C – 43°C and 13°C – 25°C respectively. During winter, frosts occur with temperatures as low as -6°C at night.

The Roodeplaat Experimental Farm is part of the Agricultural Research Council (ARC). It is located north of Pretoria and has sour mix bushveld grazing. Pretoria has a semi-arid cool climate and has an annual precipitation of 732 mm. The average maximum daily temperatures in the summer and winter is 27°C-30°C and 18°C-23°C, respectively.

3.3 Selection of animals and experimental design

The bulls used in this project (phase 1 and 2) was 10-12 months old. The number of animals for each phase can be found in table 3.1. The bulls were transported to the bull testing station on the ARC Animal Production campus in Irene, Pretoria. The treatment groups were the following: Bonsmara that was fed the high energy diet (HEB), Bonsmara that was fed the low energy diet (LEB), Nguni that was fed the high energy diet (HEN) and Nguni that was fed the low energy diet (LEN). There was ten animals per treatment. Phase 1 began in December 2015 and the animals were slaughtered in March 2016. Phase 2 started in November 2016 and was completed through slaughtering in February 2016.

Table 3.1 The number of bulls sourced from each experimental farm for phase 1 and phase 2

Experimental farm	Number of Bonsmara	Number of Nguni
Phase 1		
Roodeplaat	14	11
Vaalharts	6	9
Total	20	20
Phase 2		
Roodeplaat	13	10
Vaalharts	7	10
Total	20	20

In both phases, the cattle were dipped and dosed before being transported to Irene. They also tested negative for Brucellosis and Bovine Tuberculosis. At their arrival they received Bovine Shield Gold (2x) (Zoetis, USA), Multicloss (1x), injections of vitamins A, D and E as well as a multivitamin shot. They were dosed for internal parasites and dipped every month for ticks and external parasites (Cypermethrin). All cattle were given a 28-day adaptation period with hay fed for the first three days, followed by the low energy diet as base. The high energy diet was gradually introduced to the groups that would receive the high energy diet. After the adaptation period, they were weighed with a Weighting Indicator (Richter Scale, USA). They were randomly divided into two groups within the breeds and placed into separate pens which housed a maximum of ten animals. Water was freely available. All the pens were equipped with Calan self-feeding gates (American Calan, USA). If the animals were unable to learn how to use the Calan gates, they were removed from the group and placed separately. Four bulls from phase 2 were placed separately in pens (1 Bonsmara, 3 Nguni) and not used for sampling for phase 3.

3.4 Ingredients and composition of the diets

3.4.1 Phase 1- Diet formulation

The diets in this project was formulated in consultation with experts in animal nutrition (2016, H. Putter, Pers. Comm., Putter Voere, P.O. Box 146, Theunissen, 9410; 2016, L. Erasmus, Pers. Comm., University of Pretoria, Pretoria 002, South Africa) and feedlot diets (Coetzer, 2002). No growth stimulants were added to the diets, implanted in the bulls or given to the bulls via injections. The high energy diet was based on the traditional diet fed in feedlots. The low energy diet was based upon the diet fed to the Phase C animals of the National Beef Improvement Scheme at the ARC-AP

bull testing station. Table 3.1 lists the composition of the low energy diet compared to the high energy diet.

Table 3.2 Composition of the low energy diet and the high energy diet

Nutrients	**Low Energy Diet**		**High Energy Diet**	
	As fed	**Dry matter**	**As fed**	**Dry matter**
Dry matter %	88.4	100.0	88.4	100.0
TDN %	67.0	75.6	72.1	82.2
ME (MJ/kg DM)	**9.9**	**11.2**	**10.8**	**12.3**
Crude protein (g/kg)	130	15	130	14.3
Non-degradable protein (g/kg)	45.0	51	44.5	49
NPN (%)	15.4	17.5	15.4	17.5
Fiber (g/kg)	15.3	16.1	10.3	11.4
Roughage (g/kg)	320	350	175	199
Fat (g/kg)	30	34	33	38
Calcium (g/kg)	7.5	8.5	5.6	6.1
Phosphate (g/kg)	3.3	3.7	3.0	3.4
Ca:P	2.3:1	2.4:1	1.9:1	1.9:1
Sulphur (g/kg)	1.7	1.9	1.7	1.9
N: S	11.8:1	11.8:1	11.8:1	11.8:1

The low energy ration contained more roughage and less maize meal as compared to the high energy diet. The low energy diet had a roughage: concentrate ration of 320:380 (1:1.2), while the high energy diet had a roughage: concentrate ratio of 175:555 (1:3.2).

Feed samples were taken for composition analysis and analysed by the nutrition laboratory on ARC AP. The animals were fed ad lib after the adaptation period was completed. Feed intake was measured via Calan Gates at the bull testing station. The ingredients used in the diets are listed in Addendum A.

3.4.2 Phase 2 – Feedlot phase

The diets in phase 2 was slightly adjusted after consultation with a feedlot expert (2017, C. Coetzer, Pers. Comm., Wildswinkel) even though there was significant difference between the low and high energy diets in phase 1. Table 3.2 shows the composition of the revised diets.

Table 3.3 The composition of the low energy diet and the high energy diet used in phase 2.

Nutrients	Low Energy Diet		High Energy Diet	
	As fed	**Dry matter**	**As fed**	**Dry matter**
Dry matter %	88.0	100.0	88.4	100.0
TDN %	65.0	73.8	72.1	82.2
ME (MJ/kg DM)	**9.6**	**10.9**	**10.8**	**12.5**
Crude protein (g/kg)	130	147	130	143
Non-degradable protein (g/kg)	44.5	50.6	44.5	49
NPN (%)	15.4	17.5	15.4	17.5
Fiber (g/kg)	15.5	17.6	10.3	11.4
Roughage (g/kg)	340	386	175	199
Fat (g/kg)	30	34	33	38
Calcium (g/kg)	7.0	7.9	5.6	6.1
Phosphate (g/kg)	3.7	4.2	3.0	3.4
Ca: P	1.9:1	1.9:1	1.9:1	1.9:1
Sulphur (g/kg)	1.7	1.9	1.7	1.9
N: S	11.8:1	11.8:1	11.8:1	11.8:1

As compared to the low energy diet in phase 1, the low energy diet in phase two had a roughage: energy ratio of 340:300 (1.13:1). The diet used in phase 2 had more roughage than the low energy diet used in phase 1. The high energy diet remained the same as in phase 1. Both diets were formulated with the same ingredients (Addendum A).

3.5 Sampling and slaughter procedure

The bulls were fed for 120 days. The animals were weighed weekly and feed was weighed once every fortnight. The animals were Real Time Ultrasound (RTU) scanned with MyLabOne (Esaote, USA) (Figure 3.1) by Mr. Stephen Ratsebotsa at the commencement and at the completion of the trial. The bull was clamped in the shoot to refrain it from moving and *Helianthus* oil was used to lubricate the areas on the bull that was scanned (Figure 3.1). The P8 fat thickness was measured on the rump of the animal. Rib fat thickness, marbling as well as the eye muscle area was measured between the 12th and the 13th rib where the *Longissimus dorsi* muscle is located.

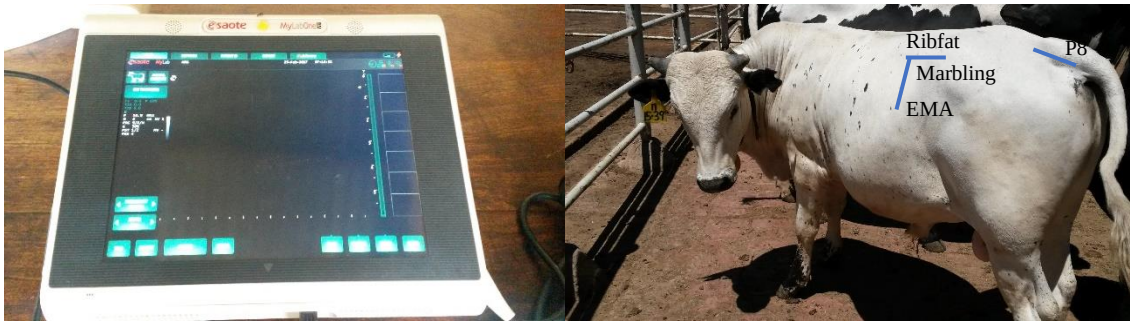

Figure 3.1 The RTU scanner, MyLabOne (Esaote, USA) (left) and the scanning positions (Marbling, Eye muscle area (EMA), Rib fat and P8 fat) on the bulls (right).

Blood collection took place at the commencement and at completion of the trial in the morning after the bulls were fasted overnight at the Bull testing centre of the ARC-Animal Production. Blood was drawn from the coccygeal vein in the tail using an 18-gauge needle and a Serum blood collection tube from Vacuette® (Greiner bio-one, Austria). The blood samples were analysed at the Onderstepoort Pathological Centre at Onderstepoort Campus, University of Pretoria for Non-esterified fatty acids (NEFA), urea, cortisol and creatinine concentration.

The bulls from phase one were slaughtered after 120 days and the bulls from phase 2 after 112 days. The animals were slaughtered over a period of 21 days due to limited capacity for slaughtering and processing of carcasses at the Irene Abattoir of ARC-AP, where all animals were slaughtered, according to the guidelines of the Society of Prevention of Cruelty to Animals (SPCA). A bolt gun is used to stun the animal in the forehead followed by cutting the jugular veins for exsanguination, and are then hoisted by the Achilles' tendon and processed.

Muscle samples were taken at the end of the trial due to ethical consideration. Muscle samples were collected from all the bulls in phase 2 for transcriptome analyses in phase 3. Liquid nitrogen was poured from a flask into a Styrofoam container. The cryotubes as well as the forceps were placed within this container to prevent heat shock. After the animal was shot, bled and hoisted, a cut was made at the dorsal right side of the animal at the 13th rib. An 8 mm biopsy punch manufactured by Miltex® (USA) was used to take samples from the cut of the *Longissimus dorsi* muscle. Forceps was used to place the muscles sample into the cryotube. The procedure was done within 10 minutes after death. After all the samples were collected, the samples were transported in the liquid nitrogen to the laboratory where it was stored in a -80 °C fridge. Two sets of samples were collected and stored in duplicate at the Biotechnology Platform at Onderstepoort and at the Genetics laboratory at ARC-AP Irene.

3.6 RNA extraction from muscle samples

RNA extraction was performed at the ARC Biotechnology Platform (ARC BTP), Onderstepoort in an RNA free laboratory. The frozen *L. dorsi* muscle samples had three replicates (each replicate

was 120 mg) for the RNA extraction from the muscle. TRIzol® Reagent (Ambion, USA) was used to extract the RNA (Figure 3.2). For the homogenization, 1.0 ml TRIzol was added as well as two ceramic beads. The sample, with the TRIzol, was placed in a GenoGrinder 2010 (SPEX sample prep, USA) for 15-20 minutes at 1730 rpm. Chloroform was added to separate the DNA and the RNA. The homogenized and separated tissue was subsequently precipitated by isopropanol. In the final step the precipitated pellet was washed with 1 ml 70% Ethanol. Each sample was treated with the RNase-free DNase set (Qiagen, Hilden, Germany) to decrease the risk of genomic DNA contamination, and it was purified with the RNeasy mini kit according to manufacturer's guidelines (Qiagen, Hilden, Germany). Extracted RNA from the samples were immediately stored in a -80 °C fridge.

Figure 3.2 The process of RNA extraction using TriZol reagent and other components.

The extracted RNA of all the samples were quantified using Qubit Fluorescent meter (Invitrogen, USA) according to the manufacturer's protocols and through gel electrophoresis with a 1% gel using Ethidium bromide and TBE.

3.7 RNA sequencing

RNA concentration was quantified with the Qubit Fluorescent meter (Invitrogen, USA) and varied between 198-570 µl/ml for a volume of 60 µl. Only 3-5 µl/ml volume was used for the RNA-seq sample preparation. RNA sequencing requires a library to be prepared before the samples can be sequenced that entails attaching an index to the samples. Sample preparation was done with TruSeq stranded mRNA protocol (Illumina, USA) (figure 3.3).

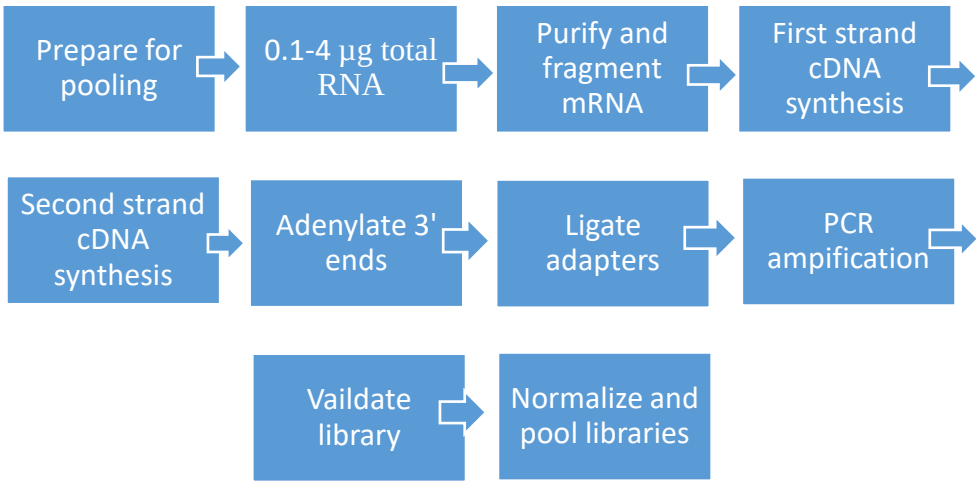

Figure 3.3 The sample preparation workflow of TruSeg Stranded mRNA protocol.

Three samples from each of the treatment groups were selected for sequencing (n=12). Three replicates from each sample was pooled and sequenced. Sequencing was performed using a HiSeq 2500 (Illumina, USA) at the ARC BTP and ran pair-ended 125 x 125. Four GB data per sample was received for downstream analyses.

3.8 Statistical analysis

3.8.1 Quantitative data

In phase one and phase two of the project quantitative data including the weight, feed intake and RTU measurements were recorded and analysed using SAS v 9.4 Proc MIXED (SAS, 2004). Fixed effects were breed, diet and origin. Proc MIXED used t-test to determine the significance with p-value set at $p < 0.05$. Feed conversion ratio (FCR) was calculated as a ratio of total feed intake (kg) to the total live weight gain over the period (kg). Average daily gain (ADG) was calculated as the average weight difference between the initial and final weights divided by the number of days the trial was run. The results of the different blood parameters were also analysed using SAS v 9.4 Proc MIXED.

3.8.2 Genomic data

The analysis of the genomic data was done on the HPC server of ARC BTP. The sequencing data generated in phase 3 of the study was analysed using various software programs. The bovine reference genome (UDM3.1.1) was the reference database. FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used to determine the quality of the data received for downstream analysis. The sliding window technique (4:10) from Trimmomatic (Bolger *et al.*, 2014) was used to trim the data and remove the adapters added in the library preparation. The average across 4 bases is used to determine if the bases should be trimmed, if the quality is below 10 the bases will be trimmed. The minimum length of the sample should not be less than 75 basepairs (bp); the sample was removed from the analyses if below minimum length. After the first quality control, it showed that the first three bases from all the samples were below the desired quality, therefore the first three bases were trimmed. Salmon (Patro *et al.*, 2017) was used to align the reads and assemble the transcripts. The output from Salmon was then loaded into R (R, 2008), where the differential gene were determined and the data was visualized using the Bioconductor (Gentleman *et al*., 2004) and DESeq2 (Love *et al.*, 2014) packages (Addendum B). The DESeq2 package uses a negative binomial distribution fitted to a general linear model to determine the differential expression of the genes and a empirical Bayes shrinkage model for fold change estimation. A gene list was generated and loaded to Panther (Mi *et al.*, 2017) to annotate the genes. Genes are deemed differentially expressed when adjusted p-value < 0.10. A fold change (fc) < 0 indicated a down-regulated gene, while an fc > 0 indicated an up-regulated gene.

Chapter 4: Results

4.1 Phase 1: Diet determination

In phase 1 the aim was to determine the suitability of the high and the low energy diet by evaluating potential differences in the traits. The average start weight for the Bonsmara was 263.84±7.43 kg and for the Nguni 212.93±6.48 kg. At the end of the trial, the Bonsmara bulls fed the high energy diet (12.5 MJ/kg) (HEB) had the highest average live weight (Table 4.1), while the Nguni bulls on the low energy diet (11.0 MJ/kg) (LEN) had the lowest live weight.

Table 4.1 The average live weight, feed conversion ratio (FCR) and average daily gain (ADG) of Nguni and Bonsmara fed two different diets with varying energy levels at the end of the trial.

Trait	Bonsmara	Nguni	Bonsmara		Nguni	
	Averages		High Energy	Low Energy	High Energy	Low Energy
Live weight (kg)	422.31^{a}±9.05	337.82^{b}±7.90	419.8^{a}±8.50	402.4^{a}±8.50	341.0^{b}±8.50	319.7^{b}±8.50
ADG	1.51±0.04	1.19±0.04	1.54^{a}±0.04	1.35^{b}±0.04	1.24^{c}±0.04	1.10^{d}±0.04
FCR	7.44^{a}±0.33	8.78^{b}±0.29	7.77^{a}±0.31	7.79^{a}±0.31	8.66^{b}±0.31	8.89^{b}±0.31

^{abcd} Different superscripts between columns differ significantly at $p<0.05$

There was a significant interaction between the diet and the origin of the two bulls with regard to live weight. The origin of the bulls had a significant effect on the performance, with both breeds from Vaalharts having a higher ADG (1.41 kg/day) compared to the bulls from Roodeplaat experimental station (1.28 kg/day).

The bulls on the low energy diet had a higher P8 fat and rib fat measurement compared to those on the high energy diet across the breeds (Table 4.2). It can be noted that the bulls that received the low energy diet had a higher marbling score than the bulls that was fed the high energy diet.

Table 4.2 The average P8 fat, rib fat, eye muscle area (EMA) and marbling for Nguni and Bonsmara bulls fed a low or a high energy diet.

Traits	Bonsmara	Nguni	Bonsmara		Nguni	
	Averages		High Energy	Low Energy	High Energy	Low Energy
P8 fat (mm)	6.17±0.18	5.36±0.15	5.60^{a}±0.16	6.38^{b}±0.16	5.01^{c}±0.16	5.63^{d}±0.16
Rib fat (mm)	3.45±0.15	2.96±0.14	3.08^{a}±0.23	3.81^{b}±0.23	2.84^{c}±0.19	3.17^{d}±0.19
EMA	53.1±2.63	49.2±2.30	52.7^{a}±2.48	50.0^{a}±2.48	45.6^{a}±2.48	51.4^{a}±2.48
Marbling	3.72±0.21	2.72±0.18	2.65^{a}±0.29	4.21^{b}±0.29	2.21^{c}±0.26	3.23^{d}±0.26

^{abcd} Different superscripts between columns differ significantly at p<0.05

For most of the traits a significant difference between the diets and the breed were observed except for the eye muscle area where there was no significant difference (p>0.05). The P8 fat measurement had a significant interaction between breed, diet and origin. Marbling had a significant difference in the breed by diet interaction (Table 4.3).

Table 4.3 The interaction between the breeds and the diets, high (H) and low (L) energy diets, of the bulls in regard to marbling score.

	Breed by Diet Effect			
Trait	**Bonsmara**		**Nguni**	
	H	L	H	L
Marbling	2.60±0.30	4.85±0.30	2.23±0.26	3.20±0.26

Significance level at p<0.05

The difference between the marbling scores due to the diets were more pronounced in the Bonsmara breed compared to the Nguni breed. The difference between the marbling score for the Bonsmara fed the two different diets and the Nguni fed the two different diets was 43%.

The Bonsmara had an average carcass weight of 226.69 kg while the Nguni had an average carcass weight of 180.11 kg. There was also a significant difference between the origins of the cattle with the bulls from Vaalharts having a heavier carcass weight (213.69 kg) than the bulls from Roodeplaat (193.11 kg). There was also a significant interaction between the diet and the origin.

In summary, there was a significant breed effect for all the traits except for eye muscle area (p< 0.05) (Table 4.4). The marbling trait also had a significant interaction (p<0.05) between the breed and the diet.

Table 4.4 Summary of the p-values and the resulting significance of the various factors in phase 1.

Factors	Live weight	FCR	ADG	P8-fat	Ribfat	EMA	Marbling	Carcass weight
Breed	***	**	***	**	*	NS	**	***
Diet	NS	NS	*	**	*	NS	***	NS
Breed*Diet	NS	NS	NS	NS	NS	NS	*	NS
Origin	**	NS	*	NS	NS	NS	NS	**
Breed*Origin	NS	NS	NS	NS	NS	NS	NS	NS
Diet*Origin	*	NS	NS	NS	NS	NS	NS	*
Breed*Diet*Origin	NS	NS	NS	*	NS	NS	*	NS

Where *** means highly significant ($p < 0.0001$); ** means moderately significant ($p< 0.005$); * means significant ($p<0.05$) and NS is not significant.

The table indicates that there was a significant difference between the diets in ADG, P8-fat, rib fat and marbling. The diets used in phase 1 can be used in phase 2 with slight adjustments as stated in chapter 3.4.2.

4.2 Phase 2

4.2.1 Performance data

In phase 2 the diets were adjusted for Metabolizable energy as described in Chapter 3 section 3.4.2. At the beginning of the trial, the Nguni had an average starting weight of 193.00±4.29 kg, while the Bonsmara had an average starting weight of 206.49±4.53 kg. In Table 4.5, feedlot measurements for the two diets analysed for the Nguni and the Bonsmara were summarized.

Table 4.5 The average live weight (kg), feed conversion ratio (FCR) and average daily gain (ADG) for Bonsmara and Nguni fed different energy levels in phase 2.

Traits	Bonsmara	Nguni	Bonsmara		Nguni	
	Averages		High energy (12.5 MJ/kg)	Low energy (10.9 MJ/kg)	High Energy (12.5 MJ/kg)	Low Energy (10.9 MJ/kg)
Live weight	337.9^{a}±5.91	303.9^{b}±5.60	354.9^{a}±5.86	330.6^{a}±5.65	301.1^{b}±5.86	306.6^{b}±5.65
ADG	1.56^{a}±0.04	1.32^{b}±0.04	1.65^{a}±0.04	1.50^{a}±0.04	1.34^{b}±0.04	1.30^{b}±0.04
FCR	7.14^{a}±0.21	8.33^{b}±0.20	6.88^{a}±0.21	7.32^{a}±0.21	8.36^{b}±0.21	8.30^{b}±0.21

^{Abcde} – Different superscripts between columns indicated significance at $p < 0.10$.

Although, the LEN had a higher live weight compared to the HEN the difference was not significant ($p > 0.10$). The breed effect on the live weight is significant with the Bonsmara having a weight of 337.87 kg compared to the 303.85 kg of the Nguni. In Figure 4.1 the weight gain trend over 5 weeks adaptation and 12 weeks of the trial was illustrated.

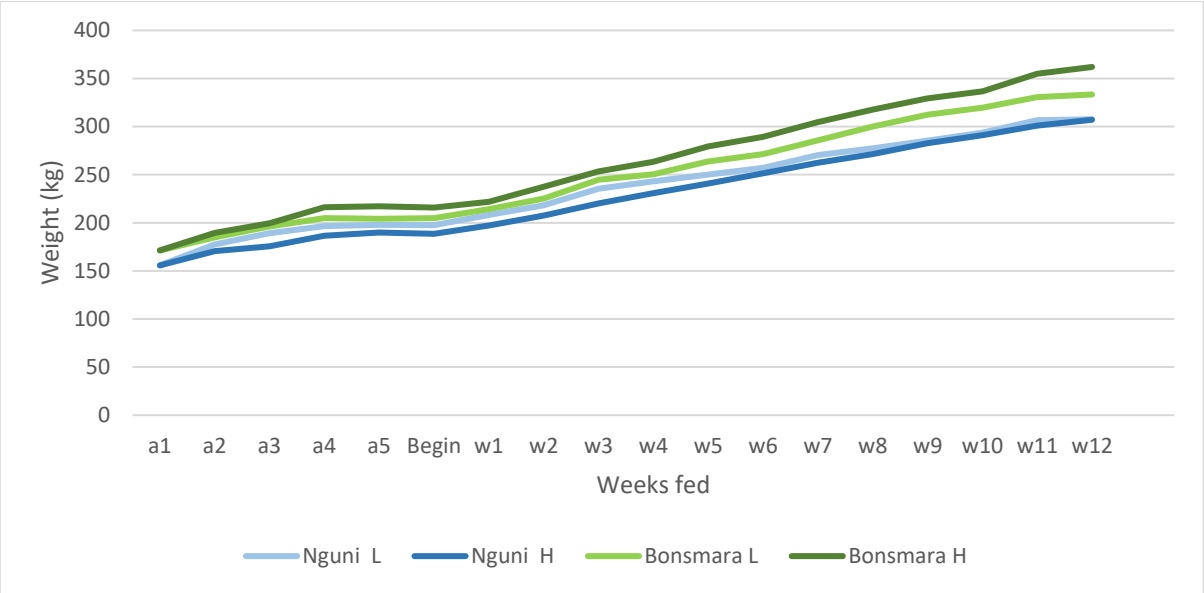

Figure 4.1 The weight gained for Nguni and Bonsmara bulls fed a low energy diet (L) or a high energy diet (H) for five weeks adaptation and 12 weeks of the trial.

Results show that the Bonsmara was the more efficient breed with regard to FCR, with a higher ADG on the high energy diet. No significant differences were observed except for breed effect as well as the interaction between breed and origin and the interaction between diet and origin.

In figure 4.2, the intake over the 12 weeks was shown with a higher intake on the high energy diet (939.24 kg) compared to the low energy diet (900.23 kg).

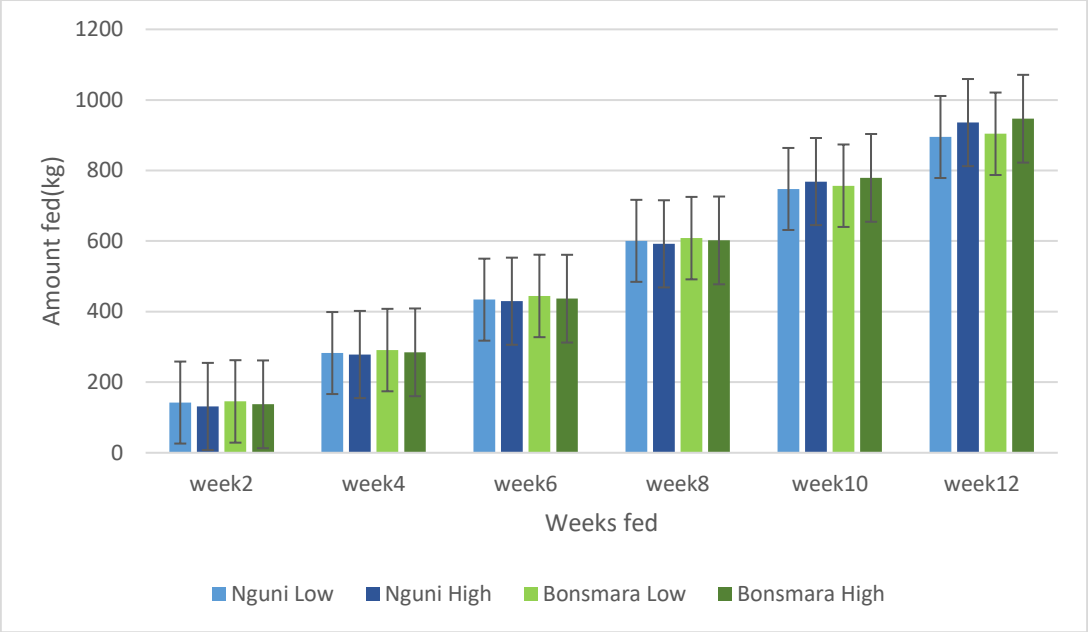

Figure 4.2 The intake of the bulls on a high or low energy diet fed over the 12-week period.

Intake was only higher from week 10. The breed as well as the diet effect were significant ($p<0.10$) om the feed intake. There was also a significant interaction between breed and origin ($p<0.05$).

4.2.2 Real time Ultrasonic scanning

Results for RTU is shown in table 4.6 with no significant differences between the diets except for marbling. The breed effect was significant for all traits ($p<0.10$).

Table 4.6 The average P8 fat, rib fat, eye muscle area (EMA) and marbling for Nguni and Bonsmara fed different energy levels in phase 2.

Traits	Bonsmara	Nguni	Bonsmara		Nguni	
	Averages		High Energy	Low Energy	High Energy	Low Energy
P8 fat (mm)	$5.5^{a}\pm0.08$	$5.3^{b}\pm0.07$	$5.3^{a}\pm0.08$	$5.6^{a}\pm0.07$	$5.3^{b}\pm0.08$	$5.2^{b}\pm0.07$
Rib fat (mm)	$3.19^{a}\pm0.13$	$2.88^{b}\pm0.13$	$3.25^{a}\pm0.13$	$3.29^{a}\pm0.13$	$2.87^{b}\pm0.13$	$2.89^{b}\pm0.13$
EMA	$57.0^{a}\pm1.07$	$54.5^{b}\pm1.02$	$57.0^{a}\pm1.07$	$57.5^{a}\pm1.02$	$54.3^{b}\pm1.07$	$54.7^{b}\pm1.02$
Marbling	3.31 ± 0.13	2.98 ± 0.12	$3.27^{a}\pm0.19$	$3.34^{b}\pm0.17$	$3.40^{c}\pm0.17$	$2.56^{d}\pm0.17$

^{Abcde} – Different superscripts between columns indicated significance at $p < 0.10$

The breed-origin interaction as well as the breed, diet and origin interactions was significantly different for P8 fat. Both breeds fed the low energy diet had more rib fat compared to the breeds on the high energy diet. This difference was however, small and not significant. The interaction between

diet and origin was significant (p<0.10). Marbling had a significant breed by diet effect. The Bonsmara fed the low energy diet had a higher marbling score (3.34) compared to the Bonsmara fed the high energy diet (3.27). The opposite was seen in the Nguni, with those that received the high energy diet having a higher marbling score (3.40) compared to those that received the low energy diet (2.56). This is in contrast to phase 2, where both breeds fed the low energy diet had a higher marbling score compared to the bulls fed the high energy diet.

4.2.3 Carcass weight

In both breeds, the bulls fed the high energy diet had a higher carcass weight than the bulls fed the low energy diet. The Bonsmara and the Nguni had an average carcass weight of 176.76 (±2.45) kg and 156.23 (±2.32 kg), respectively. The Bonsmara fed the high energy diet had the highest live weight at 187.70 (±2.43) kg. The Bonsmara fed the low energy diet had a live weight at 168.39 ±2.34 (kg). The Nguni fed the high energy diet had a live weight of 159.11 (±2.43) kg at the end of the trial. The Nguni on the low energy diet had the lowest live weight at 153.35 (±2.34). The difference between the breeds as well as across diets was significant. The interaction between breed and origin as well as between diet and origin was significant (p<0.10). In table 4.7 a summary is shown of the significance levels of the various companents and their interactions with regard to the growth and RTU of the traits

Table 4.7 A summary of the significance at various levels of p-values in the various traits

Effect	Live weight	FCR	ADG	P8 fat	Ribfat	EMA	Marbling	Carcass
Breed	**	**	**	*	NS	*	***	***
Diet	NS	NS	NS	NS	NS	NS	**	**
Breed*Diet	NS	NS	NS	NS	NS	NS	**	NS
Origin	NS	NS	NS	NS	NS	NS	NS	NS
Breed*Origin	**	NS	NS	**	NS	NS	NS	**
Diet*Origin	*	NS	NS	NS	*	NS	NS	*
Breed*Diet*Origin	NS	NS	NS	**	NS	NS	NS	NS

NS – Non- significant; * - p-value <0.10; ** p-value < 0.05; *** p-value < 0.0001

The effect of breed is significant, except in rib fat. Diet is only significant in marbling and carcass weight. None of the growth performance traits were significant for the origin of the bulls.

4.2.4 Blood components

Four blood components were analysed, namely, urea, as an indicator of the amount of protein in the bloodstream, creatine, as an indicator of the breakdown of protein, Non-esterified fatty acids (NEFA), as an indicator of the amount of lipids in the blood stream and cortisol, to measure the amount of stress the bulls experienced.

For both breeds, bulls fed the low energy diet had a higher urea concentration than the bulls fed the high energy diet (Table 4.8). There was also a significant difference between the diets ($p<0.10$) in blood urea concentration and the interaction between breed and origin.

Table 4.8 Urea, Creatine, Non-esterified Fatty acids (NEFA) and cortisol concentration from the Nguni and Bonsmara bulls fed two different energy levels in phase 2.

Traits	Bonsmara	Nguni	Bonsmara		Nguni	
	Averages		High Energy	Low Energy	High Energy	Low Energy
Urea (mmol/l)	4.04±0.21	4.9±0.19	3.6^{a}±0.20	4.2^{b}±0.20	4.7^{c}±0.20	5.1^{d}±0.20
Creatine (µmol/l)	91.22^{a}±2.79	81.80^{b}±2.53	87.1^{a}±2.65	89.6^{a}±2.68	81.6^{b}±2.65	82.0^{b}±2.68
NEFA (mmol/l)	0.11^{a}±0.02	0.12^{a}±0.02	0.16^{a}±0.02	0.1^{a}±0.02	0.1^{a}±0.02	0.1^{a}±0.02
Cortisol (nmol/l)	57.18^{a}±5.80	54.67^{a}±5.25	57.01^{a}±5.50	58.70^{a}±5.57	49.5^{a}±5.49	59.9^{a}±5.57

^{Abcde} – Different superscripts between columns indicated significance at $p < 0.10$.

The difference between the diet in regard to creatinine concentration in the blood was not significant, however the difference between the breeds and between the origins was ($p<0.05$). The concentration of NEFA in the blood had significant difference in the interaction between the diet and the origin. Table 4.9 show the significance levels of the various components and their interactions with regard to the blood parameters.

Table 4.9 A summary of the significance of various levels of the p-value in the blood components

Effect	Urea[1]	Creatine[1]	NEFA[1]	Cortisol[1]	Urea[2]	Creatine[2]	NEFA[2]	Cortisol[2]
Breed	**	NS	NS	NS	**	**	NS	NS
Diet	NA	NA	NA	NA	*	NS	NS	NS
Breed*Diet	NA	NA	NA	NA	NS	NS	NS	NS
Origin	NS	NS	*	NS	NS	**	NS	NS
Breed*Origin	NS	NS	*	NS	**	NS	NS	NS
Diet*Origin	NA	NA	NA	NA	NS	NS	*	NS
Breed*Diet*Origin	NA	NA	NA	NA	NS	NS	NS	NS

NA – not applicable; NS – non significant; * - p-value < 0.10; ** - p-value <0.05; *** - p-value < 0.0001

Superscript 1 blood taken at the beginning of the trial before the diets were fed to the bulls.

Superscript 2 blood taken at the end of the trial.

Only the breed difference in urea is constant. Diet effect is only seen in the blood urea concentration.

4.3 Phase 3 - Transcriptome analyses

4.3.1 Differentially expressed genes between the breeds

The differential gene expression analysis performed between the breeds resulted in 2214 differential expressed genes (DEG) (p-adjusted < 0.10), representing 12.94% out of the 17110 genes identified in the muscle samples of the two breeds. Of these DEG, 942 and 1272 were under expressed and higher expressed, respectively in the Nguni. In the Bonsmara, 942 genes were highly expressed and 1272 genes lower expressed. Panther (Mi *et al.*, 2017) was used to annotate 1929 genes. Most of these genes are involved in cellular processes and metabolic pathways (Figure 4.3).

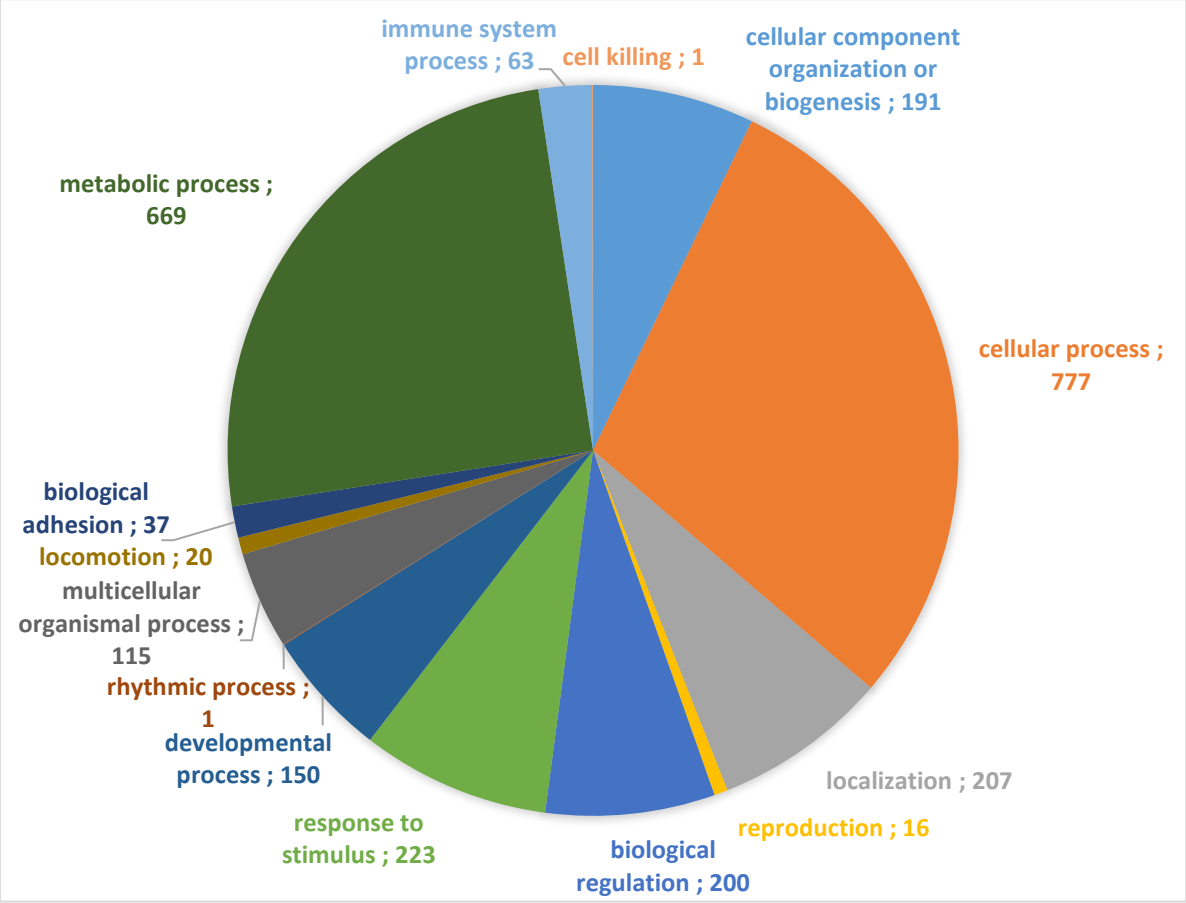

Figure 4.3 The biological pathways of the differentially expressed genes in both breeds.

A number of gene families were differentially expressed. The Ankyrin repeat domain-containing protein (ANKRD) family had five differentially expressed genes. Three and two of these

genes were upregulated in the Nguni and in the Bonsmara, respectively. The Collagen (COL) family also consists of subunits, these subunits were expressed on a higher level in the Bonsmara compared to the Nguni. However, the Proteosome (PSM) gene family were all upregulated in the Nguni. One of the Cytochrome C oxidase (COX) family was upregulated in the Bonsmara, while four was upregulated in the Nguni. The Ubiquitin carboxyl-terminal hydrolase (USP) family had three upregulated genes in the Nguni and four in the Bonsmara. In Table 4.10, the DEG of interest with < 1 has been summarized.

Table 4.10 The differentially expressed genes (p<0.05) in the Nguni (positive fc) and the Bonsmara (negative fc) breeds regardless of diet.

Gene name	Family	*log2FoldChange	p-adjusted < 0.10
ACBD7	Acyl-CoA-binding domain-containing protein 7	1.85	3.3890e-07
ADIPOQ	Adiponectin	-1.71	0.0003
ARL5B	**ADP-ribosylation factor-like protein 5B**	**2.59**	**0.0323**
ASIP	**Agouti-signaling protein**	**-3.89**	**4.0516e-05**
ATP7B	Copper-transporting ATPase 2	1.52	0.0899
CCL8	C-C motif chemokine 8	-1.74	0.0428
CDKL2	Cyclin-dependent kinase-like 2	1.55	0.0720
CELSR1	Cadherin EGF LAG seven-pass G-type receptor 1	-1.90	0.0151
CHRND	Acetylcholine receptor subunit delta	1.79	4.0975
CRAMP1	Protein cramped-like	1.75	0.0047
CRB1	Protein crumbs homolog 1	-1.74	9.7385e-05
CYP2W1	Cytochrome P450 2W1	-1.55	8.8285e-05
DNAJB13	DnaJ homolog subfamily B member 13	1.62	0.06
DPY19L3	C-mannosyltransferase DPY19L3-related	1.73	0.0992
EIF2C3	Protein argonaute-3	1.93	0.0023
ELMOD1	**ELMO domain-containing protein 1**	**-3.97**	**5.9068e-05**
FAIM2	Protein lifeguard 2	1.50	0.0403
FNIP2	Folliculin-interacting protein 2	-1.52	0.0068
FRRS1L	**DOMON domain-containing protein FRRS1L**	**2.16**	**0.0003**
FRS2	Fibroblast growth factor receptor substrate 2	1.75	0.0970

GALNT10	Polypeptide N-acetylgalactosaminyltransferase 10	1.69	0.0408
GIMAP8	GTPase IMAP family member 8	-1.55	0.0007
GLCCI1	**Glucocorticoid-induced transcript 1 protein**	**2.06**	**0.0187**
GPR142	**G-protein coupled receptor 142-related**	**2.04**	**0.0127**
GREB1	Protein GREB1	-1.74	0.0003
GSTA3	**Glutathione S-transferase A3**	**-6.85**	**0.0002**
HECA	Headcase protein homolog	1.51	0.0834
HIPK2	Homeodomain-interacting protein kinase 2	1.61	0.0012
HTR1E	5-hydroxytryptamine receptor 1E	-1.75	0.0585
INSRR	Insulin receptor-related protein	1.45	0.0590
IQCA1	IQ and AAA domain-containing protein 1	-1.52	0.0012
IQUB	IQ and ubiquitin-like domain-containing protein	1.70	0.0144
KCTD19	BTB/POZ domain-containing protein KCTD19	-1.64	0.0549
KLF12	Krueppel-like factor 12	1.53	0.0171
KLRF2	**Killer cell lectin-like receptor subfamily F member 2**	**-3.48**	**6.6278e-05**
KRT1	**Keratin, type II cytoskeletal 1**	**2.80**	**0.0996**
KRT19	Keratin, type I cytoskeletal 19	-1.46	0.0765
LEP	**Leptin**	**-2.13**	**0.0368**
LOC539009	**Ras-related GTP-binding protein A**	**2.76**	**0.0111**
LOC574091	**Nucleoside-diphosphate kinase NBR-A**	**4.15**	**4.9769e-08**
MAP3K2	**Mitogen-activated protein kinase kinase kinase 2**	**2.13**	**0.0125**
MCHR1	Melanin-concentrating hormone receptor 1	1.51	4.2401e-05
MCM8	DNA helicase MCM8	1.72	0.0208
MEGF11	**Multiple epidermal growth factor-like domains protein 11**	**2.00**	**0.0590**
MEGF6	Multiple epidermal growth factor-like domains protein 6	-1.72	0.0019

METTL21C	Protein-lysine methyltransferase METTL21C	-1.63	0.01802
MMS22L	Protein MMS22-like	1.60	0.0782
MOGAT1	**2-acylglycerol O-acyltransferase 1**	**-2.93**	**0.0011**
MORN3	**MORN repeat-containing protein 3**	**2.73**	**0.0033**
NPAS4	Neuronal PAS domain-containing protein 4	-1.81	0.0144
NR4A3	Nuclear receptor subfamily 4 group A member 3	-1.47	0.0202
OASL	**2'-5'-oligoadenylate synthase-like protein**	**-2.10**	**0.0041**
OTOS	**Otospiralin**	**-3.77**	**0.0038**
OXT	**Oxytocin-neurophysin 1**	**-2.35**	**0.0919**
PLAGL2	Zinc finger protein PLAGL2	1.88	0.0557
PRR15L	Proline-rich protein 15-like protein	1.58	0.0754
RAB6B	**Ras-related protein Rab-6B**	**-2.11**	**0.0002**
RALGAPA2	Ral GTPase-activating protein subunit alpha-2	-1.52	0.0471
RFX2	DNA-binding protein RFX2	1.82	7.5927e-05
RIIAD1	**RIIa domain-containing protein 1**	**2.13**	**0.0129**
RNASE1	Ribonuclease pancreatic	-1.48	0.0387
RUBCN	Run domain Beclin-1-interacting and cysteine-rich domain-containing protein	1.73	0.0336
RXFP1	Relaxin receptor 1	1.68	0.0095
SCAI	Protein SCAI	1.96	0.0686
SCN11A	Sodium channel protein type 11 subunit alpha	-1.89	0.0513
SENP5	Sentrin-specific protease 5	1.89	0.0006
SFMBT2	**Scm-like with four MBT domains protein 2**	**2.16**	**0.0254**
SLC1A2	Excitatory amino acid transporter 2	1.72	0.0518
SLC28A1	Sodium/nucleoside cotransporter 1	-1.79	0.0023
SLC36A4	**Proton-coupled amino acid transporter 4**	**-2.04**	**0.0558**
SMPDL3B	Acid sphingomyelinase-like phosphodiesterase 3b	-1.87	0.0434
SNAI3	**Zinc finger protein SNAI3**	**-2.20**	**3.0072e-05**

SYCP2L	**Synaptonemal complex protein 2-like**	**-3.23**	**2.2848e-05**
TAB3	TGF-beta-activated kinase 1 and MAP3K7-binding protein 3	1.73	0.0244
TMEM30C	**Cell cycle control protein 50C**	**-2.06**	**0.0144**
TMPRSS6	Transmembrane protease serine 6	-1.47	0.0519
ZBTB34	**Zinc finger and BTB domain-containing protein 34**	**2.65**	**0.0566**

*Positive log2fold change indicates highly expressed in the Nguni
Negative log2fold change indicates highly expressed in the Bonsmara
DEG in bold fc > 2

There were a number of genes of interest that did not meet the criteria for the table above as the observed fc was not higher than one, but had an adjusted p-value of less than 0.10 (Table 4.11).

Table 4.11 The differentially expressed genes of interest that had a fold change lower than one across the breeds regardless of diet.

Gene name	Family	*log2fold change	Adjusted p-value < 0.10
ACSL4	Long chain fatty acid CoA ligase 4	0.81	0.0720
ACSL6	Long chain fatty acid CoA ligase 6	-0.66	0.0274
APOBR	Apolipoprotein B receptor	-0.87	0.0188
B3GAT1	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1	-0.54	0.0362
CAPN2	Calpain	0.17	0.0764
CRYAB	Alpha-crystallin B chain	0.38	0.0554
FADS2	Fatty acid desaturase 2	-0.60	0.0270
FSTL1	Follistatin-related protein 1	-0.36	0.0007
IGFBP	Insulin-like growth factor-binding protein	-0.74	0.0716
INSIG	Insulin induced gene	-0.57	0.0230
MEF2BNB	Myocyte enhancing factor	0.26	0.0901
PRKAA	5'-AMP-activated protein kinase catalytic subunit alpha	0.42	0.0186
PRKAB	5'-AMP-activated protein kinase subunit beta-1	0.27	0.0114
PSMC	Protease regulatory subunit	0.38	0.0032
SIRT4	Sirtuins	0.41	0.0103
SIX	Homeobox protein	-0.95	0.0004

SREBF1	Sterol regulatory element-binding protein 1	-0.55	0.0304
TNNT	Troponin T, slow skeletal muscle	0.45	0.0388
USMG	Up-regulated during skeletal muscle growth protein	0.37	0.0113

*Positive values indicates expressed in the Nguni

Negative values indicates expressed in the Bonsmara

4.3.2 Differentially expressed genes between the diets across the breeds

The differential expressed analysis performed between the bulls fed the different diets resulted in 74 DEG (adjusted p-value < 0.10). This represented 0.42 % (74 out of 17619 genes). Higher expression of 31 genes were observed in all cattle fed the high energy diet compared to the cattle fed the low energy diet. Downregulation of 43 genes were observed in the bulls that received the high energy diet compared to the bulls that received the low energy diet. Sixty-two genes of the 74 were annotated and most of DEGs between the diets are involved in cellular processes and metabolic pathways (Figure 4.4).

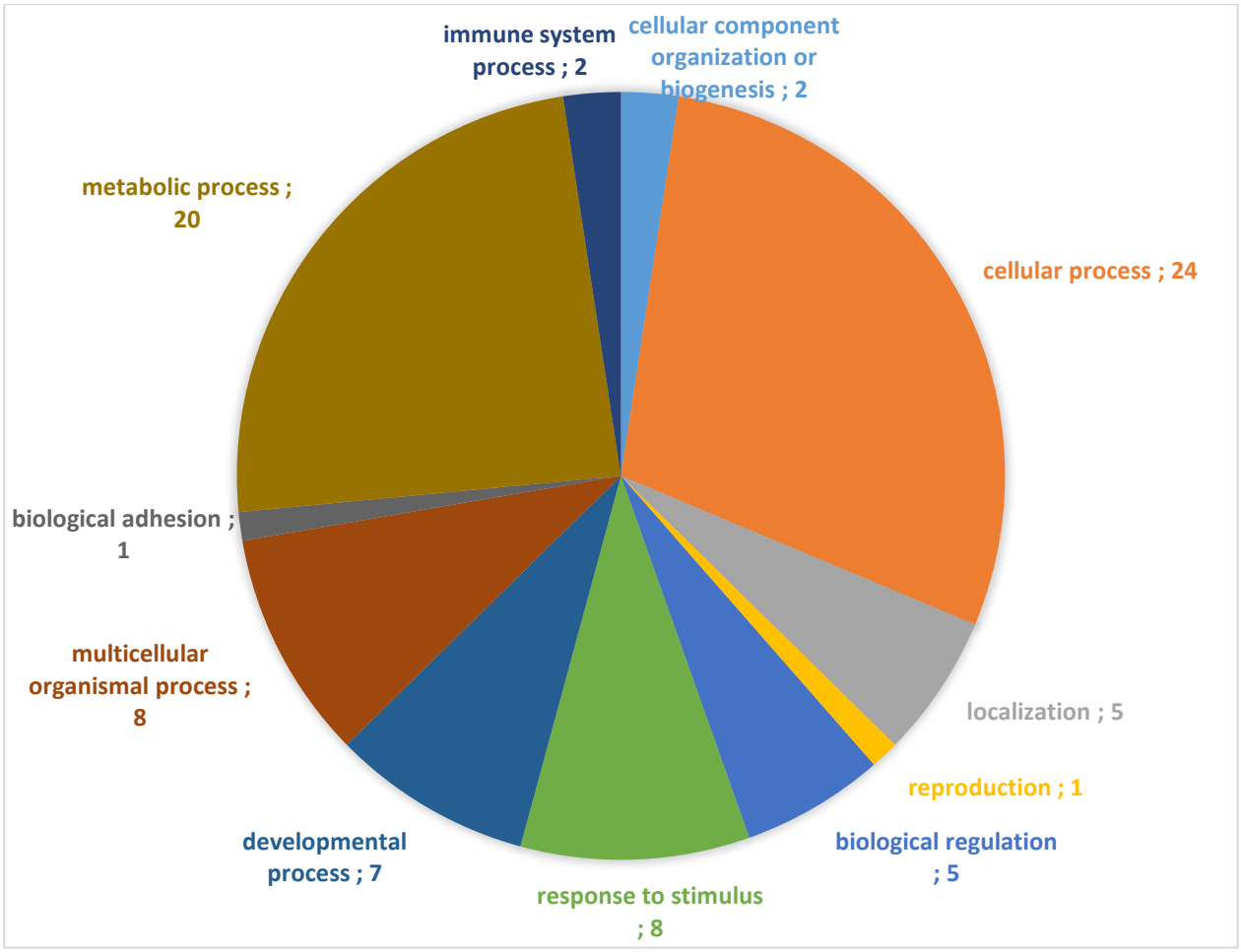

Figure 4.4 The biological pathways of the differentially expressed genes observed for the cattle fed different diets.

The differentially expressed genes mostly involved in cellular processes included SLC5A6, CHRND, TEP1, SRPK3, SPARC and OXT while PRKAG3, PSMC2, HMOX1, SIX2 and SUPT3H were involved in metabolic processes. The genes that are involved in the developmental processes included DDAH1, NET1, PAX8, PITX2, HES1, NR4A3 and CRHR2. Table 4.12 list some of the differentially expressed genes between the bulls that received the high energy diet and the bulls that received the low energy diet.

Table 4.12 Differentially expressed genes (p-value < 0.05) in the bulls fed the low energy diet (positive fc) and the bulls fed the high energy diet (negative fc).

Gene name	Family/Subfamily	*log2FoldChange	Adjusted p-value <0.10
ABCC2	**Canalicular multispecific organic anion transporter 1**	**-2.92**	**0.0309**
CHRND	Acetylcholine receptor subunit delta	-1.53	0.0070
GSTA3	**Glutathione S-transferase A3**	**5.38**	**0.0484**
HPCAL4	Hippocalcin-like protein 4	1.00	0.0185
LOC104968634	Granulysin	-1.14	0.0873
MCHR1	Melanin-concentrating hormone receptor 1	-1.21	0.0183
MTF1	Metal regulatory transcription factor 1	1.19	0.0608
NR4A3	**Nuclear receptor subfamily 4 group A member 3**	**2.13**	**0.0047**
OXT	**Oxytocin-neurophysin 1**	**-4.52**	**0.0053**
PAX8	Paired box protein Pax-8	-1.68	0.0425
PLB1	Phospholipase B1, membrane-associated	-1.06	0.0998
SLC25A33	Solute carrier family 25 member 33	1.31	0.0003
SLC5A6	Sodium-dependent multivitamin transporter	1.15	0.0001
SNAI3	Zinc finger protein SNAI3	1.53	0.0562
SUSD1	**Sushi domain-containing protein 1**	**2.96**	**0.0995**
SYNJ2	Synaptojanin-2	1.72	0.0188
TEX28	**Testis-specific protein TEX28**	**2.87**	**0.0540**

*Positive log2fold change indicates highly expressed in the bulls that received the low energy diet
Negative log2fold change indicates highly expressed in the bulls that received the high energy diet
DEG in bold fc > 2

There are some genes of interest that had a fc < 1, but was significantly different between the two diets (adjusted p-value < 0.10) (Table 4.13).

Table 4.13 The differentially expressed gene of interest that had a fold change lower than one in the bulls that received the different diets.

Gene name	Family	*log2foldchange	Adjusted p-value <0.10
ACTC1	Actin, alpha cardiac muscle	-0.86	0.0783
CRHR2	Corticotropin-releasing factor receptor 2	0.91	0.0995
KCNC4	Potassium voltage-gated channel subfamily C member 4	0.79	0.0667
LOC407241	Krueppel-like factor 15	0.72	0.0188
PITX2	Pituitary homeobox	-0.58	0.0391
PRKAG3	5'-AMP-activated protein kinase subunit gamma-3	-0.50	0.0803
PSMC2	Protease regulatory subunit	-0.21	0.0817
SIX2	Homeobox protein	-0.95	0.0216
SPARC	SPARC	0.26	0.0502
USP28	Ubiquitin carboxyl-terminal hydrolase 28	-0.69	0.0183

*Positive values indicate expressed in the Nguni

Negative values indicate expressed in the Bonsmara

4.3.3 Differentially expressed genes within the two breeds fed two diets

In the analysis for DEG between breeds and diets 3154 genes were differentially expressed between HEB and HEN (out of 17124, 18.41 %), 3584 genes between HEB and LEB (out of 16680, 21.48 %), 2244 genes between LEN and LEB (out of 16685, 13.44%) and 288 genes between LEN and HEN (out of 17068, 1.68 %). The genes that were highly differentially expressed is shown in Table 4.14. A complete list of all the DEG were attached as Addendum C.

Between the HEB and the LEB groups, 1752 genes were upregulated in the Bonsmara bulls that were fed the high energy diet (fc < 0) and 1832 genes were upregulated in Bonsmara bulls that received the low energy diet (fc > 0).

Table 4.14 The most differentially expressed genes between the breeds fed the different diets according to fold change* (in brackets).

HEB vs HEN	HEB vs LEB	LEB vs LEN	HEN vs LEN
GSTA3 (-8.89)	60s Ribosomal protein (8.22)	60s Ribosomal protein (-6.40)	GSTA3 (7.47)
Nucleolin (5.49)	OXT (-7.66)	OTOS (-5.82)	Glycine cleavage system H protein (-6.80)
60s Ribosomal protein (5.22)	Glycine cleavage system H protein (-6.56)	ASIP (-5.53)	TUBB3 (-5.23)
ELMOD1 (-4.68)	CLRN2 (5.02)	BPIFA2A (4.69)	ABCC2 (-4.05)
MOGAT1 (-4.46)	GSTA3 (4.54)	LOC574091 (4.13)	GM21964 (-3.29)
KLRF2 (-4.33)	AVP (-4.53)	GSTA3 (-3.99)	MAD2A (3.75)
TUBB3 (4.19)	Histone H3.1 (-3.87)	TUBB3 (-3.95)	CALY (3.13)
AVP (-4.16)	FAM131B (-3.31)	KRT1 (3.68)	C5AR1 (3.02)
GPR142 (3.98)	SLC36A4 (-3.22)	SNAI3 (-3.59)	GRB7 (-2.58)
CLRN2 (3.94)	NR4A3 (3.13)	LWFIKKN1 (-3.55)	CHRND (2.62)

*Positive log2fold change indicates highly expressed in the Nguni or in the bulls fed the low energy diet
Negative log2fold change indicates highly expressed in the Bonsmara or in the bulls fed the high energy diet

PPARGC1A (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and SIX2 (Homeobox protein SIX2) did not have a fold change of more than 2, but are deemed as genes of interest. Some gene families were also differentially expressed. The Collagen family had seven genes upregulated in the HEB group and two genes upregulated in the LEB. Two genes of the Cytochrome C oxidase (COX) family were upregulated in the HEB and three genes in the LEB. Of the Forkhead box protein (FOX) family, one gene was upregulated in the HEB and four in the LEB. All the differential expressed genes of the Insulin-like growth factor (IGF) family were upregulated in the HEB group (IGF2, IGFBP4, IGFBP5, IGFBPL1), while those of the Insulin-induced gene (INSIG1, INSIG2) family was upregulated in the LEB group. The myocyte-specific enhancer family had one DEG in the HEB (MEF2D) and one in the LEB (MEF2A). The PRKAG family had one gene upregulated in the Bonsmara's fed the high energy diet (PRKAG3) and four upregulated in the LEB (PRKAA1, PRKACA, PRKACB, PRKAG2). The sirtuin family had 3 DEGs, one was upregulated in the HEB (SIRT2) while the other two were upregulated in the LEB (SIRT1, SIRT5). There were genes of interest that had a fold change lower than one; Calmodulin (CALM), Calpain (CAPN11), Pro-opiomelanocortin (POMC) and Proteosome assembly chaperone (PSMG).

There were 118 genes upregulated in the Nguni bulls that received the high energy diet (HEN) (fc < 0) and 170 genes upregulated in the Nguni bulls that received the low energy diet (LEN) (fc > 0). Paired box protein 8 (PAX8) had a fold change of more than 2. PITX2 (Pituitary homeobox 2) and

TUBB3 (Tubulin beta-3 chain) were upregulated in the HEN but had a fold change lower than 2. The following genes were upregulated in the LEN with a fold change lower than two, C28H10ORF1165 that codes for an adipogenesis regulatory facor, LOC407241 (Kreuppel-like factor 15), POMK (Protein O-mannose kinase), PSMC31P (Homologous-pairing protein 2) and SPARC.

In the groups, HEB and HEN, 1332 genes were upregulated in the Bonsmara bulls that received the high energy diet (fc < 0). The number of genes that were upregulated in the Nguni bulls fed the high energy diet were 1822 (fc >0). The genes that follow are differentially expressed (adjusted p-value < 0.10), but has a fold change lower than 2. All the genes of the Collagen family that were differentially expressed between the HEB and the HEN groups were upregulated in the HEB. This is also seen in the IGF family (IGF2, IGFBP4, IGFBP5) as well as the NADH-ubiquinone oxidoreductase family (MT-ND3, MT-ND4, MT-ND4L, MT-ND6). The cytochrome C oxidase family had two genes upregulated in the HEB and 6 genes in the HEN. Four of the FOX family were upregulated in the HEN, while one was upregulated in the HEB (FOXP4). All the DEGs from the 5'-AMP-activated protein kinase catalytic family was upregulated in the HEN group (PRKAA1, PRKACB, PRKAG2). This is similar to the Proteasome family (9 DEGs). Adiponectin (ADIPOQ), Apolipoprotein B receptor (APOBR), Calpain (CAPN11), Cystatin, DNA methyltransferase 1 (DNMT1), POMC and Homeobox proteins (SIX2, SIX5) were upregulated in the HEB. The genes of interest that were upregulated in the HEN are the following: Calmodulin (CALM2, CALM3), Acetylcholine receptor (CHRNB1), INSIG2, PITX2, POMP, SIRT4, SIRT5 and Up-regulated during skeletal muscle growth protein 5 (USMG5).

There were 937 upregulated genes in the Bonsmara fed the low energy diet (LEB) (fc < 0) and 1307 genes that were upregulated in the Nguni fed the low energy diet (LEN) (fc > 0). Alpha-2-antiplasmin (SERPINF2) was upregulated in the Nguni fed the low energy diet and had a fold change higher than 2. All five Cytochrome C oxidase genes were upregulated in the LEN group. The IGF family had two genes upregulated in the LEN group (IGFBPL1, IGFLR1) and one in the LEB group (IGFBP3). Two genes from the 5'AMP-activated protein kinase family were upregulated in the LEN group (PRKAB1, PRKAG3) and one in the LEB group (PRKACA). All three genes of the homeobox protein family (SIX1, SIX4, SIX5) were upregulated in the Bonsmara's that received the low energy diet. The following genes of interest was significant but did not have a fold change higher than 2. The genes that were upregulated in the LEB were: FOXN2, FSTL1, PITX2 and PPARGC1A. The genes that were upregulated in the Nguni's that received the low energy diet were ADIPOR2, CALM, CALM2, POMT1, POMT2, SIRT2 and SNAI2. In Figure 4.5, the expression of genes associated with growth and muscle deposition were illustrated.

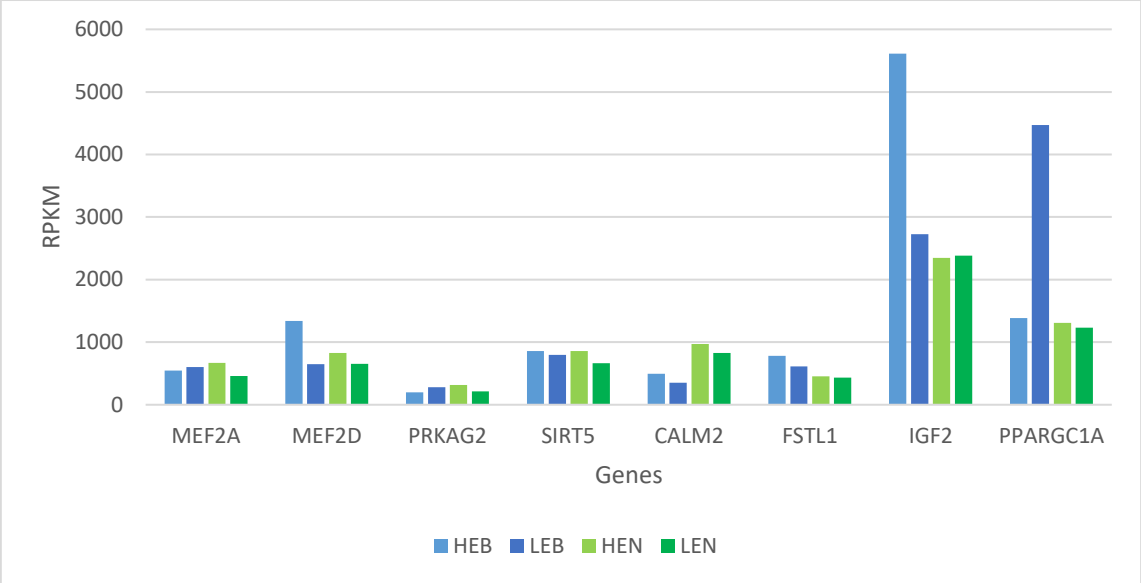

Figure 4.5 The differential expression of genes of interest (fc <1) in the four treatment groups.

In this study these genes of interest had a fold change lower than 1, with MEF2A and MEF2D only differentially expressed between HEB and LEB. The gene, PPARGC1A was differentially expressed between LEB and LEN and between HEB and LEB. There are however a number of genes that did have a fold change > 2 (Figure 4.6).

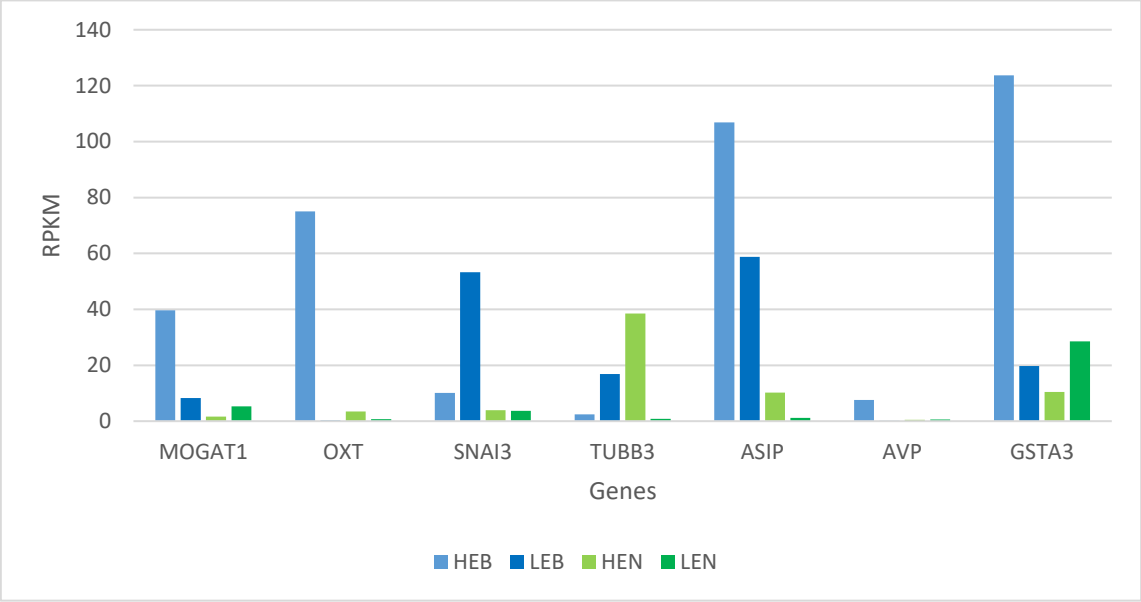

Figure 4.6 Differentially expressed genes of the breeds fed the two different diets.

Across the treatment groups GSTA3 was the most differentially expressed with a fold change of 4 and higher. TUBB3 was also differentially expressed across the treatment groups, however the fold change between HEB and LEB was lower than between the other groups (HEB vs. HEN, HEN

vs. LEN and LEB vs. LEN). A heat map depicting the relationship between the bulls is shown in figure 4.7.

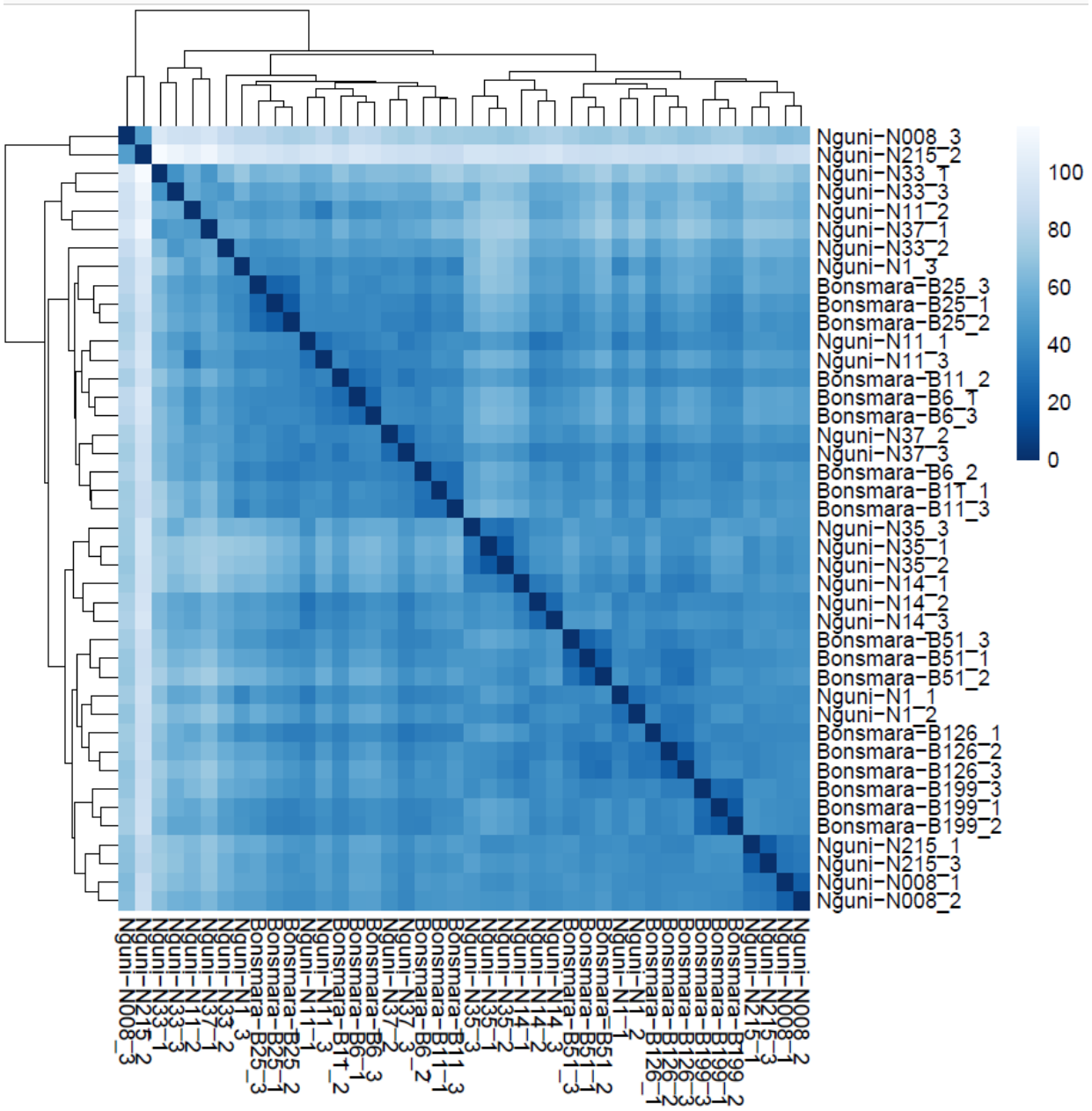

Figure 4.7 Heatmap of the relationship between the bulls.

The heatmap shows that there was little difference between the bulls. There was no significant trend between the two origins, the breeds and the diets.

Chapter 5: Discussion

5.1 Introduction

The demand for animal protein is increasing as the world population increases (Cassar-Malek *et al.*, 2008). It is estimated that by 2050 the world population will increase to 9 million inhabitants and most of the population growth is expected to take place in the developing world including Africa (UN DESA, 2017). South Africa is expected to have 75.7 million people by 2050. The amount of beef produced from the veldt is not sufficient to meet this demand for meat as a result of limited arable land (Niemann *et al.*, 2011). Often the veldt in South Africa is not sufficient to meet the requirements for finishing weaner calves due to overgrazing and drought conditions. Therefore, feedlots will play a more important role in the future.

The indigenous Nguni is predominantly used in extensive production systems, in both commercial and communal systems. As the Nguni is a small frame animal, feedlots tend to discriminate against them (Dugmore, 2014), as the small frame cattle breeds deposit proportionally more fat compared to the larger frame breeds. Traditionally, feedlot diets consists mostly of maize resulting in a high energy diet (Coetzer, 2002). As the Nguni is adapted to the veldt of South Africa, it could be possible that a diet lower in energy could lead to more optimal production in feedlots. There was a need to explore this as the Nguni breed is found across South Africa. Communal as well as emerging farmers choose to farm with Nguni cattle as this breed is hardy and fertile (Mapiye *et al.*, 2009). These farmers need a market for their weaners.

The identification of differentially expressed genes can be used to study gene expression associated with production traits and genes contributing to quantitative variation between livestock breeds (Wang *et al.*, 2009). The RNA-seq technique uses transcript abundance with high sensitivity to identify the differential expressed genes, even those genes with low transcript abundance (Marioni *et al.*, 2008). The aim of this study was to determine if a low energy diet is more suitable compared to the high energy diet for finishing Nguni cattle under intensive feedlot conditions. Various approaches have been applied to complete this aim. Firstly, a diet low in energy was formulated that was significantly different from the high energy diet. Secondly, a feedlot trial was used to gather data on the performance of the bulls. Performance traits were recorded and analysed as well as RTU traits. In the trial blood was analysed for urea, creatinine, cortisol and NEFA concentration. Lastly, muscle samples from the feedlot trial was used for gene expression analysis to study DEG between the breeds fed low or high energy diets.

5.2 Diet determination (phase 1) and feedlot trial (phase 2)

In the diet determination phase of this project; twenty Nguni and twenty Bonsmara bulls were fed two diets as described in Chapter 3. There were significant differences ($p<0.05$) between the breeds for live weight, feed efficiency, average daily gain, rump fat (P8 fat), rib fat, marbling as well as carcass weight. This was expected as breed differences have been established in previous studies (Du Plessis & Hoffman, 2007). An unexpected result from the diet determination phase was that the bulls across the breeds fed the low energy diet had a higher fat content (measured as rump fat, rib fat and marbling) compared to the groups that received the high energy diet. This was unexpected as the high energy diets provide large amounts of propionate that is converted to glucose, resulting in lipogenesis (Gilbert *et al.*, 2003; Joseph *et al.*, 2010). In contrast, Bauman & Griinari (2003) reported that diets low in energy decrease the ruminal pH, resulting in changes to the biohydrogenation pathway and a suppression of the Sterol Regulatory Element Binding Transcription Factor (SREBF1) gene as well as the Peroxisome Proliferator-Activated Receptor Gamma (PPARG) gene. The suppression of these genes leads to a decrease in lipid synthesis (Obsen *et al.*, 2012). In a study by Teixeira *et al.* (2017) the animals fed a higher energy diet had a lower ruminal pH compared to the animals fed the low energy diet. In reaction to the lower pH the microbiota and the biohydrogenation pathways changed and resulted in a decreased expression of the SREBF1 gene. The consequence of this is reduced lipid synthesis. Studies done by Brandebourg & Hu (2005) and Jenkins & Harvatine (2014) also support this result. In the current study, significant differences between the diets were found in the average daily gain, rump fat, rib fat and the marbling score of the carcasses. This indicated that the effect of the diet was suitable and was used in the phase 2 feedlot trial with minor adjustments.

In the feedlot phase (phase 2) Nguni fed the low energy diet had a slightly higher live weight (306.6 kg) compared to the Nguni fed the high energy diet (301.1 kg), however this was not significant. In another study, the Nguni's final weight was on average 338 kg, which is higher than what was found in this project (Scholtz, 2010). It has been reported that Nguni gained proportionally more weight compared to Angus or Bonsmara under harsh extensive conditions, such as the Northern Cape climate, but produced low carcass weights (107 kg) (Muchenje *et al.*, 2008).

In this study the breed effect was significant ($p<0.05$) for live weight, but diet effect was not significant for live weight. Carcass weight had a breed and a diet effect. The Bonsmara fed the high energy diet had the highest live weight, carcass weight, ADG and better FCR between the groups. This result was expected, as the Bonsmara is a medium frame breed (Wheeler *et al.*, 1996) while the indigenous Nguni is a small frame cattle breed. Du Plessis & Hoffman (2007) also found a breed effect on live and carcass weight with Simmental crosses having the heaviest weights and Nguni the lightest. The carcass weight of the bulls fed the high energy diet was higher than the group that was fed the low energy diet. Carcasses from diets that are high in energy are heavier and contain more fat than carcasses from cattle fed diets that mostly consists of forage (du Plessis & Hoffman, 2007; Frylinck *et al.*, 2013). For all traits in phase 2 no origin effect was observed ($p>0.05$).

In this study the FCR of the Nguni was poorer than reported in various studies (Schoeman 1989; Scholtz, 2010). There was a significant difference ($p<0.05$) between the breeds and the diets with regard to the feed intake. The low energy diet is a bulkier diet and therefore has lower feed efficiency and lower feed intake compared to a high energy diet. As this diet is bulkier the rumen fills quickly, the animal feels satiated and is less likely to continue eating. The high energy diet is more efficiently converted and absorbed and therefore the feed intake of a high energy diet will be higher compared to the feed intake of a low energy diet. Bonsmara has a larger frame than the Nguni and therefore need more energy to maintain their size and to grow. More feed therefore needs to be consumed to maintain their higher energy requirement. Strydom (2008) reported that the Nguni compared favourably to a number of late maturing breeds with regard to feed efficiency. There is a significant difference ($p<0.05$) between the breeds for the FCR, but not between the diets. There was no significant difference in phase 2 between the breeds or the diets for ADG.

Various articles reported that feeding a high energy diet to animals would lead to higher energy and fat deposition resulting in a higher P8-fat and rib fat measurement (May *et al.*, 1992; Schaake *et al.*, 1993; Prior *et al.*, 1977). This is in line with the results of the feedlot trials (phase 2) of this study. Schoonmaker *et al.* (2004) found that feeding a high concentrate (high energy) diet caused an appreciable amount of energy to be partitioned towards subcutaneous fat deposition, thereby accelerating the physiological maturity. The Bonsmara had a higher P8-fat and ribfat measurement than the Nguni, which can be attributed to different breed types. Subcutaneous fat makes a greater contribution to adiposity in large framed breed types (Cianzio *et al.*, 1985). Koch *et al.* (1979) and Wheeler *et al.* (1996) reported breed differences in rib fat at a constant weight. In contrast, Camfield *et al.* (1999) observed higher rib fat percentages for smaller framed breed type steers when compared to the larger framed beef type steers. This was not observed in this project, as the Bonsmara, which is a medium frame breed, had more rib fat compared to the Nguni. A study by Sprinkle *et al.* (1998) reported no significant differences in rib fat among large and small framed steers when their access to a maize-based diet was limited, but the differences between the breeds were significant when the steers had ad libitum access to the diet. It was also observed in this project that differences between the breeds become less obvious on lower energy diets as also reported by du Plessis & Hoffman (2007). The second largest fat depot in the body is the subcutaneous fat depot (Cianzio *et al.*, 1985). This depot may consume more energy than the intramuscular fat depot, which causes subcutaneous fat to be deposited faster than intramuscular fat. However, Sanga cattle have a capacity to deposit more fat intramuscularly compared to *Bos taurus* cattle and crosses, as those breeds tend to deposit fat subcutaneously (Shaffer *et al.*, 1981). These differences in fat deposition can have implications for fatty acid mobilisation in terms of thermoregulation and energy reserves (Nonoka *et al.*, 2008) and could therefore be seen as mechanisms that can be used to adapt to the environment. The difference in P8-fat between the breed-diet-origin interaction in this study was significant ($p<0.05$).

Marbling refers to the amount of intramuscular fat between bundles of muscle fibres (Hocquette *et al.*, 2010). It is a measure of the quality of the meat. The bulls fed the high energy diet had a higher marbling score compared to the bulls fed the low energy diet. This is similar to the findings of Crouse *et al.* (1984) where the high energy diet (grain-based diet) had a higher marbling score (4.31) than the low energy diet (roughage-based diet) (3.60). Diets high in energy increase intramuscular fat deposition (Crouse *et al.*, 1984; Joseph *et al.*, 2010). Strydom *et al.* (2000) found that the Bonsmara had a higher marbling score than the Nguni, which is in accordance with this study. Between different breed types, marbling can differ in the amount, the structure as well as the distribution of the fat in the muscles (Albrecht *et al.*, 2006). The difference between the breed and the diet was significant ($p<0.05$), as well as the interaction between them.

Blood urea is an indicator of the rumen ammonia concentration (Hayashi *et al.*, 2005). It is also related to intake and solubility of Nitrogen-containing compounds. There was a significant ($p<0.05$) breed effect in the concentration of urea both before and after the diets were fed to the bulls. The Nguni had a higher blood urea concentration (4.87 mmol/l) compared to the Bonsmara (4.04 mmol/l) which is not in accordance to the results of a study done by Mapiye *et al.* (2010 a) where it was found that the average blood urea concentration from Nguni cattle were lower (2.3 mmol/l) than that of crossbred cattle (2.8 mmol/l). However, the study done by Mapiye *et al.* (2010 a) focused on animals raised on pasture where the diet consists of grazing and lick, while the present study focused on feedlot animals where the diet was controlled. Scholtz & Lombard (1984) also found that the Bonsmara had a lower urea concentration (2.36 mmol/l) than the Nguni (3.38 mmol/l). The blood urea concentration found in this study is in accordance to a study done by Ndlovu *et al.* (2007) where the urea concentration of the Nguni was 4.9 mmol/l. The urea concentration in the blood is inversely correlated to the efficiency of N utilization (Nonoka *et al.*, 2008). The decrease is generally associated with an increase in N utilization efficiency (Butler *et al.*, 1996). The observed high urea concentration in Nguni in the present study might be an indicator that the Nguni bulls utilised amino acids less efficiently for growth and development. The diets also had a significant difference ($p<0.10$) where a higher urea concentration was found in the blood of the bulls fed the low energy diet. Therefore, the bulls utilised the N more efficiently when fed the high energy diet than when fed the low energy diet.

The blood creatinine concentration of bulls in this study was lower than observed by Mapiye *et al.* (2010 a) and Ndlovu *et al.* (2007). Mapiye *et al.* (2010 a) found that the Nguni had a creatinine concentration of 115.8 µmol/l and Ndlovu *et al.* (2007) a concentration of 107.2 µmol/l, whereas in the present study the Nguni had a creatinine concentration of 81.8 µmol/l. This might be as a result of the feedlot feeding of the present study compared to that of the pasture grazing of the two studies mentioned. However, Doornenbal *et al.* (1988) set the minimum value for cattle creatinine at 10 µmol/l, which is much lower than the creatinine concentration found in this study. The Nguni cattle

had a lower creatinine concentration compared to the Bonsmara, which is in accordance to the results of Mapiye *et al.* (2010 a). Creatinine is a product of the breakdown process of creatine-P and its rate of production depends on the body muscle mass (Gross *et al.*, 2005) and could explain the higher creatinine concentration observed in the Bonsmara in this study compared to the Nguni cattle. The Bonsmara is a medium frame breed and therefore has a higher intake and more skeletal muscle growth than the small frame Nguni (Adachi *et al.*, 1997).

Non-esterified fatty acids (NEFA) concentration in the blood is an important indicator of the energy status of beef cattle (Agenas *et al.*, 2006). The NEFA concentration in this study (0.125 mmol/l) was lower than the NEFA concentration found in the study of Mapiye *et al.* (2010 b) with 0.25 mmol/l. The study by Mapiye *et al.* (2010 b) was however performed using natural grazing while the present study focussed on feedlot diets. In this study, the bulls fed the lower energy diet had a lower NEFA concentration (0.099 mmol/l) compared to the bulls fed the high energy diet (0.137 mmol/l). As NEFA is an indicator of the energy status of the cattle, these results were expected. However, the Nguni (0.125 mmol/l) had a higher NEFA concentration compared to the Bonsmara (0.112 mmol/l). The Bonsmara is expected to have a higher NEFA concentration as it is a medium frame breed type and therefore need more energy for maintenance. No literature could be found that studied blood parameters in the feedlot context. NEFA is released into the blood circulation as a direct result of lipid catabolism (Ndlovu *et al.*, 2007). The use of adapted small frame cattle genotypes is recommended under low-input production systems where there is limited energy supply. Low NEFA concentration could indicate a lower energy demand or a lower amount of breakdown of adipose tissue. In contrast to the present study, Ndlovu *et al.* (2009) and Shaffer *et al.* (1981) observed a low NEFA concentration in the Nguni cattle and concluded that the Nguni could have low energy requirements and low energy demands as an adaptive mechanism. Mapiye *et al.* (2010 b) states another possible explanation as that the Nguni breed tends to deposit fat intramuscularly instead of subcutaneously and could have less subcutaneous fat as energy reserves that could be mobilised to meet the energy requirement compared to the crossbred.

5.3 Transcriptome analysis (phase 3)

In the third phase of this project the transcriptome analysis was performed on the muscle samples of the bulls. Firstly, the two main factors were compared; breed corrected for diet and diet corrected for breed. Thereafter, the four treatment groups were compared with each other. The four treatment groups were the Bonsmara bulls that received the high energy diet (HEB), the Bonsmara bulls that received the low energy diet (LEB), the Nguni bulls that received the high energy diet (HEN) and the Nguni bulls that received the low energy diet (LEN).

It was expected that there would be a greater breed effect compared to the diet effect in this study when the differentially expressed genes were analysed. There were 2214 differential

expressed genes between the Nguni and the Bonsmara regardless of diet and only 74 differentially expressed genes between the bulls that received the high energy diet and the bulls that received the low energy diet.

5.3.1 Differential expressed genes in Bonsmara and Nguni

Leptin, the Agouti-signalling protein (ASIP) and Oxytocin-neurophysin (OXT) were differentially expressed between the Nguni and the Bonsmara. All three of these genes were elevated in the Bonsmara. As Leptin, ASIP and OXT are involved in appetite control, it is to be expected that the genes' expression would be elevated in the medium frame breed, which require more energy for maintenance, compared to the small frame breed. The Agouti-signalling protein is also implicated in marbling (Albrecht *et al.*, 2012; Mizoguchi *et al.*, 2010; Sadkowski *et al.*, 2014) as it is predominantly expressed in adipocytes (Sumida *et al.*, 2004). The marbling correlation was also observed in this study, as the Bonsmara had the higher marbling score compared to the Nguni and the gene expression was elevated in the Bonsmara. The Agouti gene is a member of a gene network that could influence marbling development by regulating intracellular Ca^{2+} concentration. It stimulates a key enzyme, fatty acid synthase, in lipogenesis (Zemel *et al.*, 2004). The Agouti gene was also expressed higher in the LEB compared to the LEN. The Agouti gene is supported by the elevated expression of DNA methyltransferase (DNMT3B) in the Bonsmara compared to the expression in the Nguni. The DNA methyltransferases are thought to be responsible for somatic tissue development, fat deposition, ribeye area (Guo *et al.*, 2012) and is well known as epigenetic regulators (Turek-Plewa & Jagodzinski, 2005). Another gene that was expressed higher in the Bonsmara is the SNAI3 gene, which is part of a DNA binding transcription factor family of genes. This gene, SNAI3, is in direct competition with myoD (Kataoka *et al.*, 2000) and therefore blocks myoD from binding. This results in a decrease of myogenic differentiation (Soleimani *et al.*, 2012). As SNAI3 and several genes that are involved in fat deposition was elevated in the Bonsmara, it might suggest that the Bonsmara had reached physiological maturity while the Nguni was still growing, which is contrary to the general perception of the industry. As an animal reaches physiological maturity it does not deposit muscle further, but only deposits fat. The Bonsmara had higher rib fat and P8 fat compared to the Nguni. This suggestion is strengthened by the elevated expression of MOGAT in the Bonsmara. Monoacylglycerol acytransferase (MOGAT), also known as MGAT, encodes an enzyme that catalyzes the synthesis of triglycerides from 2-monoacylglycerols and acyl-CoA. All the family members of MOGAT pathway are viewed as high priority candidate genes for quantitative traits related to dietary fat uptake, triglyceride synthesis and storage in livestock (Winter *et al.*, 2003). The MOGAT pathway is essential for dietary fat resorption and is an important biosynthesis of cellular triacylglycerol as it generates diacylglycerol (DGAT). In the Nguni, mitogen activated protein kinase 2 (MAPK2) was upregulated. The signalling pathway to which this

gene belongs are associated with lipid or muscle metabolism and adipose tissue differentiation (Chen *et al.*, 2015). This supports the observation that the Nguni might not have reached physiological maturity at the time the trial ended.

5.3.2 Differentially expressed genes between the diets across the two breeds

Diets are a key source of variation and transcriptomics can be used to identify functional candidate genes and pathways influenced by nutrition (Tizioto *et al.*, 2016). PAX8 as well as PITX2 genes involved in myogenesis were upregulated in the bulls that received the high energy diet. Myogenesis requires specific transcription factors, such as PAX3 (Goulding *et al.*, 1999) and these genes are precursors of the muscle regulatory factors (MRF) that leads to myogenin expression and muscle growth. PAX3 is correlated with PITX2 (Shih *et al.*, 2007) and expressed in all cells where these genes are present. The pathway to which PITX2 belongs, controls myoblast growth by regulating the expression of the crucial genes that control the G1 cell cycle. Growth factor-dependent signalling results in the release of PITX2-associated corepressors (Baek *et al.*, 2003). Despite playing a crucial role in myogenesis in the embryo, PITX2 can be found in most muscles of the adult animal (Shih *et al.*, 2007) where it seems to play a role in the maturation or maintenance of muscle cells. As these genes were upregulated in the bulls that received the high energy diet, it can be suggested that the high energy diet being fed to the animals result in a heavier live weight. This is in line with the phenotypic results that were obtained in the feedlot trial of this project. The carcass weight was significantly higher ($p<0.05$) in bulls fed the higher energy diet compared to the bulls fed the low energy diet. The bulls fed the high energy diet had a higher rump fat (P8) and rib fat measurement compared to the bulls that received the low energy diet across both breeds. Schoonmaker *et al.* (2004) found that feeding a high concentrate (high energy) diet caused an appreciable amount of energy to be partitioned towards subcutaneous fat deposition.

In this study, the bulls fed the high energy diet had a higher marbling score than the bulls fed the low energy diet. The difference between the diets were significant ($p<0.05$) for marbling, as well as the interaction between the breeds and the diets. This is similar to a study by Crouse *et al.* (1984). Diets high in energy increase intramuscular fat deposition. Intramuscular fat has been shown to develop within connective tissue alongside myofibres (Moody & Cassens, 1968). Wang *et al.* (2009) suggests that a prerequisite for intramuscular fat development might be the expansion of the extracellular matrix (Tahara *et al.*, 2004). In this study, the SPARC gene was upregulated in the bulls that received the low energy diet. The SPARC gene influences the synthesis and interaction with the extracellular matrix. However, the bulls that had a higher marbling score were those that received the high energy diet. As the SPARC gene was upregulated in the bulls that received the low energy diet it might suggest that at a later stage the bulls might have deposited more

intramuscular fat given time. Sanga cattle have a capacity to deposit more fat intramuscularly compared to *Bos taurus* cattle and crosses, that tend to deposit fat subcutaneously (Shaffer *et al.*, 1981). These differences in fat deposition can have implications for fatty acid mobilisation in terms of thermoregulation and energy reserves (Nonoka *et al.*, 2008) and could therefore be viewed as mechanisms for adaptation to the environment. The higher expression of CRHR2, CHRND as well as Kreuppel-like factor 15 in the bulls that were fed the low energy diet strengthens this observation, as both are associated with higher marbling score and adipogenesis (Wibowo *et al.*, 2007). The NR4A3 gene also strengthens this observation as it was expressed higher in the bulls that received the low energy diet. In a study done by Lin *et al.* (2017), there was a correlation with the expression of NR4A3 and intramuscular fat deposition in goats.

In ruminants, the majority of glucose is derived from propionate via gluconeogenesis (Young, 1977) and are associated with high energy diets. In a study of Baldwin *et al.* (2012) with the inclusion of propionate in the ration for steers, it was found that pathways that was mainly influenced by the increased energy in the ration included lipid metabolism, small nucleotide biochemistry, carbohydrate metabolism and molecular transport. Similar pathways were observed in the present study. However, neither PPAR nor SREBF was differentially expressed in this study between the bulls that received the high energy diet and the bulls that received the low energy diet. The transcription factors PPAR and SREBF are some of the main genes involved in lipid metabolism. Other studies have found that a high energy diet could reduce the expression of the Sterol Regulatory Element Binding Transcription Factor (SREBF1) and Peroxisome Proliferator-Activated Receptor Gamma (PPARG) genes, which consequently reduces lipid synthesis (Bauman & Griinari, 2003; Obsen *et al.*, 2012). This mechanism can possibly reduce the degree of marbling found in the meat.

In this study, a breed effect was found in the differential expression of SREBF. The Bonsmara had a higher expression of SREBF1 and SREBF2 compared to the Nguni. The expression of SREBF1 is generally elevated in cells that are depleted of lipids (Rawson, 2003). Elevated expression of SREBF1 is consistent with enhanced adipogenesis. This also strengthens the suggestion that the Bonsmara had reached physiological maturity as mentioned above. Angus bulls, in a study by Teixeira *et al.* (2017), had greater levels of SREBF1 expression which resulted in greater percentages of intramuscular fat.

The SREBP-1c regulate gene activation by binding to sterol regulatory element sequences present in the enhancer/promoters of each gene, including SCD1 gene (Shimano, 2001). PPARG is a transcription factor essential for adipogenesis (Obsen *et al.*, 2012), and a reduction in its expression, as well as that of SREBF1, may decrease the expression of other genes related to fat biosynthesis (Vyas *et al.*, 2014). The expression levels of SCD1 genes were greater in the muscles of Nellore bulls fed the lower energy diet and lower in Nellore bulls fed the higher energy diet (Teixeira *et al.*, 2017) while in this study the LEB had a higher expression of SCD3 compared to the LEN. Stearoyl-CoA desaturase (SCD) is regarded as an indicator of terminal adipocyte differentiation

(Martin *et al*., 1999). The higher expression of SCD in the LEB probably indicates the greater number of mature adipocytes per unit muscle compared to the LEN. According to Smith *et al.* (2009), diets with high energy concentrations are responsible for the production of meat with a high degree of marbling, as intramuscular fat tissue appears to be more sensitive to insulin than subcutaneous adipose tissue. Thus, ingredients that increase propionate production, such as maize, which increase the metabolizable energy content of the diet, have greater glycogenic and insulinogenic capacities, which would increase intramuscular fat deposition (Gilbert *et al*., 2003).

5.3.3 Differentially expressed genes within the two breeds fed two diets

A higher number of genes were differentially expressed between the LEB and the HEB, compared to the LEN and the HEN. This may suggest that the Nguni is more adaptable regarding diet quality as the Nguni is found in regions with variation in grazing quality. Juszczuk-Kubiak *et al.* (2014) attributed the muscle characteristic differences in gene expression found between Polish Holstein-Friesian and Limousine to be the consequence of metabolic and physiological differences in these breeds that have evolved in response to production purposes. The variation in total energy requirements of animals of the same species and under similar management circumstances may arise from differences in physiological processes (Johnson *et al.*, 2003). The main selection criteria for the Nguni may have been hardiness and adaptablitity, while the focus in the Bonsmara is fast growth. A study done by Bongiorni *et al.* (2016) also found that selection for breeding objectives changed the expression of some genes. Bongiorni *et al.* (2016) found that the Italian Maremmana breed was selected for robustness in extensive production systems in harsh environments similar to the Nguni. On the other hand, the Chianina breed was selected for fast growth rate and conformation such as the Bonsmara. Analysis of the transcriptome leads to the identification of specialized biological functions and regulatory genes which would be used as the selection of markers for further breed improvement programs (Sodhi *et al.*, 2014).

One of the highly differentially expressed genes in this study was glutathione-s transferase alpha 3 (GSTA3) that was differentially expressed in all the groups having a fold change of -6.85 higher expression in the Bonsmara compared to the Nguni and 5-fold higher expression in the bulls that received the low energy diet compared to the bulls that received the high energy diet. The enzyme encoded by GSTA3 is used as a steroidal isomerase to convert the adrostenedione to the precursor of testosterone (Dourado *et al.*, 2014; Hayes *et al.*, 2005). Steroidogenesis is the physiological and developmental processes that occur due to the actions of steroid hormones. The GSTA3 gene is expressed highly in tissues where steroidogenesis occurs or tissues that are susceptible to the effect of steroidogenesis. All members of the glutathione-s transferase family detoxify several substances by catalysing their products to glutathione. It is found throughout the body, but is found in the highest concentration in the testis (Benbrahim-Talaa *et al.*, 2002; Hayes *et*

al., 2005). Steroids, such as testosterone and estradiol, are commonly used in the feedlot to promote muscle mass for heavier carcass. No steroidal treatments were given to the bulls in the feedlot trial. The Bonsmara had the heavier carcass and GSTA3 was expressed higher in the breed, however the bulls that received the high energy diet had a heavier carcass weight, but GSTA3 was downregulated in the muscle. It is not clear why GSTA3 was upregulated in the bulls that received the low energy diet. However, Hou *et al.* (2012) linked GSTA3 to lipid metabolism. No literature could be found on the role of GSTA3 in the muscle of animals. Another steroidogenesis gene, Testis-specific protein 28 (TEX28), was also expressed 2-fold higher in the bulls that received the low energy diet compared to the bulls that received the high energy diet.

Arginine vasopressin-neurophysin (AVP) was expressed 4-fold higher in the HEB compared to the LEB. These genes upregulate myogenin expression by enhancing the expression of MEF2 transcription factors (Scicchitano *et al.*, 2002) and by stimulating phospholipase D activity in myoblasts (Naro *et al.*, 1997). AVP is a myogenic differentiation factor of significant importance as it acts by inducing and activating MEF2 transcription, which regulated myogenin expression, without affecting the expression of myogenic-differentiation-inducers, such as insulin-like growth factors. It was also expressed 4-fold higher in the HEB compared to the HEN. As HEB had the highest live weight as well as carcass weight it could be expected that myogenic differentiation factors would be higher expressed than in the other treatment groups. Oxytocin-neurophysin (OXT) was expressed higher in the bulls that received the high energy diet compared to the bulls that received the low energy diet. It was also elevated in the HEB compared to the LEB. These genes help regulate responses to stress and injury as well as growth and development, nutrient absorption and energy metabolism (Gimpl & Fahrenholz, 2001). It also influences feeding behaviour by increasing feed intake (Bjorkstrand & Uvnas-Moberg, 1996). This can be explained by noting that the low energy diet is mostly roughage and that a ration based on roughage leads to rumen fill. This leads to the animal feeling satiated. Tubulin beta 3 (TUBB3) is viewed as a potential candidate gene related to proteolysis (de Oliveira *et al.*, 2014). Tubulin expression was elevated in LEB and in HEN compared to LEN and HEB. This might indicate that LEB and HEN had higher rates of proteolysis.

Muscle that is converted to meat is the foundation of the beef industry (Hocquette *et al.*, 1998). The high feed price in beef cattle production means that profitability depends on the productive and efficient use of feed for maintenance and growth with low excesses and losses (Nkrumah *et al.*, 2006). Muscle energy metabolism can be assessed by dividing the nutrients between oxidative and non-oxidative pathways (Hocquette *et al.*, 1998). This includes the storage of nutrients, the efficiency of ATP production and utilization and for muscle functions the balance between ATP supply and requirements. There are many genes that are involved in uptake and intracellular metabolism of glucose, but insulin is probably the major regulator (Hocquette *et al.*, 1998). Other factors such as calcium release, which stimulate glycogen breakdown by increasing the activity of phosphorylase is

also involved. Insulin-like growth factor (IGF2) has been associated with various myogenic effects. It has been associated with an increase longissimus muscle area (Sherman *et al.*, 2008), eye muscle area (Goodall & Schmutz, 2007), post-natal growth, metabolism and body weight regulation (DeChiara *et al.*, 1990).

In this study, IGF2 was expressed higher in the HEB compared to the LEB and the HEN. This is in line with the phenotypic results as the HEB had a higher live weight than the rest of the groups. The binding factor were also expressed higher in the HEB compared to the other groups. In the LEB, IGFBP3 was also expressed higher compared to the expression of the gene in the LEN. The IGF2 gene maps close to quantitative trait loci (QTL) for hot carcass weight (Casas *et al.*, 2004) and marbling score (MacNeil & Grosz, 2002). Insulin-like growth factor binding protein (IGFBP2) regulates the availability of IGF1 and IGF2 (Jones & Clemmons, 1995). As with IGF2, Pro-opiomelanocortin (POMC) was expressed higher in the HEB compared to the LEB and the LEN. This is also in accordance with the phenotypic results as the carcass weight was higher in the HEB than in the LEB. This gene is also associated with hot carcass weight (Buchanan *et al.*, 2005) and with rib-eye muscle-related traits (Gill *et al.*, 2010).

Contrary to IGF2 and POMC, MEF2A was expressed higher in the LEB and in the HEN compared to the HEB. However, MEF2D was expressed higher in the HEB compared to the LEB. This fold change is however negligible. The Myocyte-specific enhancer factor 2 (MEF2) family of transcription factors bind directly to the promoters or enhancers of a majority of muscle-specific genes (Lilly *et al.*, 1995) and interact with members of the MyoD family to activate skeletal muscle differentiation during myogenesis (Zhoa *et al.*, 2011). The MEF2 family serve as a target for calcium-dependent signalling to drive oxidative and slow-fiber specific genes (Chin *et al.*, 1998). The family is predominantly expressed in cardiac and skeletal muscles (Pollock & Treisman, 1991). Elevated MEF2 expression is observed during postnatal skeletal muscle growth and regeneration (Bachinski *et al.*, 2010).

Pituitary homeobox 2 (PITX2) and PAX8 was expressed higher in the HEN when compared to the HEB and the LEN. However, when the PAX8 expression was compared between the HEB and the HEN, a higher expression was observed in the Bosmara indicating higher muscularity. This gene is a downstream target of growth factor signalling pathways that control cell-type specific growth (Kioussi *et al.*, 2002). It is involved in the PITX2 pathway that regulates the G1 cell cycle control gene expressions to control the proliferation of myoblasts. This gene is expressed in almost all muscle in the adult animal and cells that expressed PAX3 (Shih *et al.*, 2007). It plays a role in the maturation and maintenance of muscles. The predominant proteasome differentially expressed in this study was PSMC31P. It was expressed higher in the LEB compared to the HEB and expressed higher in the LEN compared to the LEB and in the HEN. As PSMC is expressed higher in the LEN, it might indicate that given time the ADG and rump fat thickness of this group might have increased. Proteosomes (PSMC) is involved in the regulation of the cell cycle, differentiation and apoptosis (Wu

et al., 2004). It is associated with average daily gain, average daily feed intake and backfat thickness (Guo *et al.*, 2008). The ubiquitin-proteosome system is an important intracellular protein degradation pathway and plays a role in protein turnover. The function of the ubiquitin carboxyl-terminal hydrolase (USP) transcription factors is to bind sites that are required for muscle-specific gene expression (Sartorelli *et al.*, 1990). Various genes of this family were differentially expressed in this study. Most were expressed higher in the bulls that received the high energy diet.

The metabolism of skeletal muscle has major influences on meat quality as it influences structural and biological characteristics of the muscle (Roux *et al.*, 2006) by influencing meat tenderness (Hovenier *et al.*, 1993). It should be noted that in this study, muscle and not intramuscular fat samples were used for the transcriptomic analysis.

Three genes namely ACTL8, TNNT3 and TPI1 was highly expressed in the HEB that had the highest marbling score compared to the rest of the groups. The MDH1 gene was expressed higher in the LEB and HEN when compared to the HEB. Actin (ACTA1) and troponin (TNNT) are involved in skeletal muscle fiber development, while triosephosphate isomerase (TPI1) and malate debydrogenase (MDH) are involved in metabolic processes such as glucogenesis and carbohydrate metabolism (Chang *et al.*, 1993; Davenport *et al.*, 1991; Sasaki *et al.*, 2014). The TPI1 gene also play a role in intramuscular fat deposition (Kim *et al.*, 2009; Sasaki *et al.*, 2005) while Shin & Chung (2016) found that ACTA1, TNNT1 and MDH2 genes were highly expressed in the low marbling group and only TPI1 was highly expressed in the high marbling group compared to the low marbling group. In contrast to Shin & Chung (2016), ACTL8 and TNNT3 was expressed higher in the high marbling group (HEB) of this study. However, MDH was expressed in the low marbling group (LEB and HEN) and TPI was expressed in the highly marbling group (HEB) which is similar to what Shin & Chung (2016) reported. The ACTA1 gene encodes skeletal muscle alpha-actin (Clarke *et al.*, 2007) which forms the core of the thin filament of the sarcomere in skeletal muscle and has the function to prevent actin-myosin interaction in resting muscles (Fink *et al.*, 2008). This indicates that the HEB group was still depositing muscle, however as the TPI gene was also highly expressed, the HEB might have reached physiological maturity. Animals with a high muscularity and high glycolytic activity display a reduced development of intramuscular fat (Hocquette *et al.*, 2010). It was reported by Wang *et al*. (2005) that several genes associated with energy metabolism were highly expressed in muscle of Japanese Black cattle, which is known for its highly marbled meat.

Adipocytes regulate energy homeostasis through appetite controlling and insulin sensitivity or by storing excess energy as triglycerides and oxidizing the triglycerides during energy deprivation. In comparison of the expression between the HEB and the LEB, FOXO1, SIRT1 and SIRT5 was expressed higher in the HEB. The SIRT2 gene was expressed lower in the LEB compared to the other groups. Two Sirtuins (SIRT4 and SIRT5) was also expressed higher in the HEN when compared to the HEB. The FOXO1 gene is a member of the forkhead transcription factor class O

family and is involved in adipocyte differentiation (Nakae *et al.*, 2003) as well as myoblast proliferation (Kousteni, 2012). This gene family regulates master transcription factors such as PPARGC1A (Corton & Brown-Borg, 2005; Kousteni, 2012), the expression of myostatin and contribute to the control of muscle cell growth and differentiation (Allen & Unterman, 2007). The SIRT1 gene regulates the activity of FOXO1 (Brunet *et al.*, 2004) and SIRT2 supresses adipogenesis by deacetylating FOXO1 to promote binding to PPARG (Wang & Tong, 2009). The SIRT1 gene is expressed throughout preadipocyte differentiation (Gui *et al.*, 2016; Liu *et al.*, 2014). A higher expression of FOXO1 and SIRT1 can be observed during adipocyte differentiation (Nakae *et al.*, 2003). This indicates higher adipocyte differentiation in the HEB. Mammalian SIRT4 gene plays an important role in regulating mitochondrial gene expression and fatty acid oxidation in muscle cells (Ahuja *et al.*, 2007). This gene represses fatty acid oxidation in nutrient-replete conditions and promotes lipid anabolism (Laurent *et al.*, 2013). As SIRT4 was expressed higher in the HEN compared to HEB, it could indicate a higher rate of lipid anabolism in the HEN.

In the current study, PPARGC1A was expressed higher in the HEB compared to the LEB and in the LEB when compared to LEN. When glucose levels are low, activation of PPARG in muscle through genes like SIRT1 and PPARGC1A increase fatty acid oxidation, glucose uptake and mitochondrial biogenesis (Sevane *et al.*, 2013). The PPARGC1A pathway plays a crucial role in the transcriptional regulation of hepatic gluconeogenesis genes that are activated at fasting and inhibited by SREBP1 in a fed state (Yamamoto *et al.*, 2004). The gene from the wingless signal transduction pathway (SFRP5) was expressed higher in the LEN when compared to the LEB, indicating a higher potential for marbling (Wang *et al.*, 2009). A family member, SFRP2 was expressed higher in the LEB than in the HEB. Wang *et al.* (2009) reported that mitochondrial genes (MTCYB, COX7A2, MTND4, MTND4L) were highly expressed in animals with a low marbling potential. The HEB had a higher expression of MTCYB, COX, MTND4, MTND4L compared to the HEN. However, MTND3 was expressed higher in the LEN compared to HEN. Intramuscular fat deposition within muscle can be associated with fatty acid oxidation and adenosine triphosphate (ATP) synthesis arising from muscle tissue (Pethick *et al.*, 2005). Jurie *et al.* (2007) reported that intramuscular fat content was correlated with cytochrome c oxidase (COX) and Kim *et al*. (2009) found ND2 and COX3 were also involved in the mitochondrial oxidative phosphorylation pathway and reported increased expression of these genes resulted in higher ATP production. A previous study (Kim *et al.*, 2008) also indicated that ND1 is correlated to higher intramuscular fat content in *longissimus* muscle.

In the current study, COX1, COX3, ND1, ND2 and ND5 are expressed on a higher level in the HEB compared to the HEN. This may suggest that there was higher ATP production in the HEB. However, COX7C was expressed higher in the HEN compared to the HEB. Baldwin *et al.* (2012) also found that the steer that received a higher energy diet had an elevated COX7C expression level. Contrasting to this, COX3 was expressed higher in the LEN compared to the HEN. This suggests that the LEN had more ATP production. As both the HEN and the LEN had elevated COX levels, it

could be that the Nguni overall had a higher ATP production. However, when the two breeds were compared regardless of diet, COX was not differentially expressed between them. Kim *et al.* (2009) also showed that ND2 and COX3 could be linked with intramuscular fat content. This is similar to the HEB as it had the highest marbling score, but contrasting to this the LEN had the lowest marbling score. The mitochondria also play a role in fat synthesis (McKay *et al.*, 2003). These results and supporting results from Kim *et al*. (2009) indicate that oxidative metabolism in the mitochondria might be linked to intramuscular fat content and fatty acid utilization for triacylglycerol synthesis. As mitochondrial oxidative phosphorylation is involved in the generation of energy, such as ATP, Wang *et al.* (2009) indicated that compared to the high marbling animals, the animals with low marbling potential may use more energy to support more rapid growth of muscle during the period to weaning, when fractional rates of muscle growth are increased (Lehnert *et al.*, 2007). Gardner *et al.* (2007) reported that animals sired by high muscular genotypes have greater oxidative enzyme activity in muscles and therefore store less energy in adipose tissue. In the highly marbled animals, the expression of several extracellular protein genes are similar to that of adipogenic-related genes (Wang *et al.*, 2009).

Collagen is a major structural protein in skeletal muscle extracellular matrix (Miao *et al.*, 2015). The majority of the collagen family was expressed higher in the HEB, which is similar to the results of Baldwin *et al.* (2012). Adiponectin (ADIPOQ) was expressed higher in the HEN than in the HEB. However, SPARC, the gene that influences the synthesis and interaction with the cellular matrix was only significantly differentially expressed in the LEN compared to the HEN. Intramuscular fat has been shown to develop within the perimysium connective tissue alongside myofibers (Moody & Cassens, 1968). Perimysium is a major connective tissue in muscle and contains collagen fibers. The expansion of the extracellular matrix may be a prerequisite for intramuscular fat development (Wang *et al.*, 2009). This is also seen in studies done by Nishimura *et al.* (1999) and Tahara *et al.* (2004). An increase in expression of INSIG1 provides a feedback signalling mechanism to restrict both lipogenesis and adipogenesis (Graugnard *et al.*, 2009). INSIG1 and INSIG2 was expressed higher in the LEB compared to the HEB. However, INSIG2 was also expressed higher in the HEN compared to the HEB. Therefore, lipogenesis and adipogenesis was higher in HEB compared to LEB and HEN.

Insulin effectively stimulates muscle glucose oxidation and adipogenesis (Kokta *et al.*, 2004; Rosen & MacDougald, 2006), partly through upregulation of IRS1 transcription (Tseng *et al.*, 2005) and through activation of downstream signalling cascades including transcription factors (SREBF1), nuclear receptors (PPARGC) and their gene targets. Nguni's fed the low energy diet had an elevated expression of MMP15 compared to HEN and HEB had a higher expression of MMP11 than HEN. The matrix metalloproteinase (MMP) gene plays a role in collagenolysis and has been implicated in extracellular matrix breakdown (Lauer-Fields *et al.*, 2002).

In this study, SERPINF2 was upregulated in the LEN compared to the LEB and IGFBP3 was upregulated in the LEB group compared to the LEN. The gene SERPINF2 was down-regulated during adipocyte differentiation (Burton et al., 2004). As IGFBP3 was upregulated in the LEB, adipocyte differentiation was increased (Mizoguchi *et al.*, 2010). This suggests that a higher rate of adipocyte differentiation could be found in the LEB. In the other treatment groups, SERPINE1 was upregulated in the HEB group compared to the HEN.

Tenderness was beyond the scope of this study, however various genes have been reported to have an influence on this meat quality trait. Marbling influences the tenderness of the meat (Hausman *et al.*, 2009; Wang *et al.*, 2005) and has already been discussed above. There are various other factors that also influence the tenderness such as the calpain system. Calpain 11 (CAPN11) and Calmodulin (CALM) was highly expressed in the HEB compared to the LEB and the HEN. Calpain (CAPN) enzymes are responsible for protein breakdown in meat whereas calpastatin is an inhibitor of calpains. Patel & Lane (1999) reported that calpains regulate adipocyte differentiation. However CALM3 was expressed higher in the LEB compared to the HEB. In the HEB, PRKAG2 was expressed less than compared to the LEB and the HEN. Calmodulin (CALM) genes are linked to calpastatin activity in the muscle, while PPM2C and PRKAG2 are linked to calpain muscle enzyme activity. Family members of PPM2C, PPM1A and PPM1F was expressed higher in the LEB compared to the HEB. The interaction between CALM and CAPN influences meat tenderization. Dunner *et al.* (2013) reported an association between the calpain/calpastatin gene-network system and meat texture.

The PRKAG3 gene was expressed higher in the HEB compared to the LEB. It was also expressed higher in the LEN compared to the LEB. The PRKAG3 gene encodes AMP-activated protein kinase (AMPK) and is involved in the regulation of AMPK activity in skeletal muscle and strongly influences glycogen metabolism. Glycogen content in muscle is correlated to meat quality in livestock as it influences post-mortem maturation process and the ultimate pH. The AMP-activated protein kinase (AMPK) has been pointed out as one of the main actors in the regulation of intracellular energy metabolism (Carling, 2004). Once AMPK is activated, it stimulates both an increase in fatty acid oxidation and an increase in glucose uptake to meet the energy demands of the working muscle (Winder, 2001). Numerous mechanisms of AMPK action on lipid and carbohydrate metabolism have been proposed (Ferre *et al.*, 2003; Hardie *et al.*, 2003). Encoded by the PRKAG3 gene is the γ3-peptide that is one of the three γ-isoforms for the γ-regulatory subunit of AMPK and shows muscle specific expression (Cheung *et al.*, 2000). Li *et al.* (2012) found an association between PRKAG3 gene and the tenderness trait. Other genes that are implicated in influencing tenderness include KCNK3 and ACTC1 that was over expressed in tough meat and PAX7, OXT and CHRND that was overexpressed in tender meat (Bongiorni *et al.*, 2016).

5.4 Conclusion

The diet determination phase of this study showed that there was significant differences between the high and the low energy diet according to the phenotypic traits. The diets were therefore used in phase 2, the feedlot trial. A breed effect was expected as the Nguni is a small frame breed type while the Bonsmara is a medium frame breed type. A diet effect was only seen in the marbling trait as well as in the carcass weights of the bulls. A diet effect was observed in the transcriptomic analysis, with bulls that was fed the low energy diet having a higher potential for marbling. Genes that are associated with marbling were upregulated in the bulls that received the low energy diet compared to the bulls on the high energy diet. Diet had a greater effect on the Bonsmara compared to the Nguni according to transcriptomic results. Genes associated with muscle deposition was expressed higher in the Bonsmara compared to the Nguni.

This study is the first of its kind to be performed in South Africa. It can serve as a baseline for differentially expressed genes in the muscle of South African cattle in the feedlot.

Chapter 6: Critical review & Recommendations

The main goal for a sustainable beef industry is to produce a desirable carcass composition while maintaining efficient growth. Balancing nutritional efficiency, animal health and reducing negative environmental impact is also of increasing importance for sustainability. Understanding nutrient utilization in animals can lead to improvements in nutrients use efficiency and product quality. This can be done by studying the transcriptomic changes in productive tissues and service function tissues in response to dietary influences.

Results from this study indicate that at the end of the trial the Nguni cattle had not finished growing. In the transcriptomic study, several fat deposition genes were upregulated in the Bonsmara compared to the Nguni. These genes include Leptin, ASIP, OXT, DNMT3B and MOGAT. The SNAI3 gene that inhibits myogenic differentiation was also elevated in the Bonsmara compared to the Nguni. This suggests that at the end of the trial, the Bonsmara cattle had reached physiological maturity but the not the Nguni cattle. This is in contrast to other literature. This suggestion is further strengthened by the elevated expression of MAPK2 in the Nguni compared to the Bonsmara. A further factor is that several genes that influence marbling (SIRT, ND, COX, ADIPOQ, SERPINF2) were elevated in the Nguni, but the phenotypic value of marbling score was lower than what is implied by the gene expression. This leads to the conclusion that the Nguni needed more time to reach physiological maturity. This is contradictive of the perception of the industry, that the Nguni deposits fat too early in comparison with exotic or crossbred breeds. Sanga cattle, which includes the Nguni, tend to deposit fat intramuscularly compared to exotic and crossbred cattle. This could be an adaptation mechanism where Nguni deposit fat first. It seems, however, after the sufficient fat has been deposited, the Nguni begins depositing more muscle. This might be the reasoning for backgrounding (grazing an animal on pasture and feeding it a supplement to gain weight in preparation for the feedlot), commonly practised in the Nguni breed.

The varying level of energy in the diets (low energy vs. high energy) seems to result in different components being deposited. Various genes (PITX2, PAX8, Leptin, AVP, OXT) was upregulated in the bulls that received the high energy diet compared to the bulls that received the low energy diet. These genes influence muscle deposition and therefore, lead to a heavier carcass. This is also seen in the phenotypic results, where the bulls that received the high energy diet had higher live as well as carcass weights. However, genes that influence intramuscular fat deposition (SPARC, CRHR2, CHRND, NR4A3, MMD) was elevated in the bulls that received the low energy diet compared to the bulls that received the high energy diet. This was not shown in the phenotype, as the bulls that received the high energy diet had a higher marbling score. Extension of the feeding period may provide a chance for the gene expression in the bulls that received the low energy diet to show in the phenotype. This observation, where feeding a high energy diet did not lead to higher intramuscular fat deposition has been seen in various studies.

Greater gene expression differences could be seen in the Bonsmara between the bulls that received the high energy diet and the bulls that received the low energy diet compared to the differential gene expression in the Nguni between the bulls that received the high energy diet and the bulls that received the low energy diet. The diet effect therefore was more pronounced in the Bonsmara compared to the Nguni. This transcriptomic observation can be validated by the phenotypic values. The Nguni did not have significant differences in performance values between the bulls that received the high energy diet and the bulls that received the low energy diet. On the contrary, the Nguni bulls that received the low energy diet had a slightly higher live weight compared to the Nguni bulls that received the high energy diet. The carcass weight did differ a bit more, but less difference in carcass weight compared to the Bonsmara's on the high and low energy diets. The Nguni is found across South Africa, in communal and commercial production systems, and is therefore adapted to the fluctuating feed quality. Nguni's tend to gain proportionally more weight compared to exotic or crossbred breeds under harsh conditions. These gene expression differences based on different breeding goals has been observed in other breeds.

6.1 Recommendations

There are a few genes that holds potential for further investigation in future studies. One of these genes is glutathione s-transferase (GSTA3) that is known for its steroidal influences and a precursor to testosterone. It can be argued that this gene may influence muscle deposition similar to testosterone, however no confirming literature could be found. It has been suggested that this gene is linked to lipid metabolism. In the Bonsmara, this gene was elevated compared to the expression in the Nguni, however, it was also elevated in the bulls that received the low energy diet. This gene was the most differentially expressed gene in this study. Another gene that is involved in reproduction, but is also highly differentially expressed between the groups is testis-specific protein 28 (TEX28). This gene was elevated in the bulls that received the low energy diet compared to the bulls that received the high energy diet. No literature could be found that explained the role of TEX28 in the muscle. It could be viewed similar to GSTA3 having the same influence. Tubulin beta 3 (TUBB3) is a candidate gene for proteolysis, but further explanations of its role in muscle could not be found. These genes should be included in future studies.

As indicated, the physiological state of the animal changes as gene expression changes. It is therefore recommended that further trials are performed with an extended feedlot period with samples taken at different time points. This could assist to determine the mechanism for adipogenesis and lipogenesis in the Nguni and the ideal feedlot period if the Nguni is to be fed the low energy diet. Exotic breeds, such as the Angus, other indigenous breeds such as the Afrikaner and *Bos indicus* breeds such as the Brahman should also be included in further studies. It will also

be interesting to measure gene expression of grass fed Nguni cattle to compare to the Nguni in the feedlot fed the low energy diet. A nutrigenomics approach should be used in future studies.

In conclusion, there was a greater breed effect compared to the diet effect throughout the trial, seen in both the phenotypic values as well as the transcriptomic results. The variation in energy levels of the diets, however, did have a larger effect on the Bonsmara compared to the Nguni.

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Addendum A: Ingredients of the diets

Table A.1 Ingredients used in the low energy diet compared to the high energy diet of phase 1

Ingredients	Low Energy diet (As fed)	High Energy diet (As fed)
Hominy chop	70.0	60.0
Maize meal	310.0	495.0
Brewer's grain	150.0	120.0
Molasses	80.0	80.0
Urea (feed grade)	7.0	8.0
Cotton OCM (26)	40.0	40.0
Buffer (Sodiumbicarb)	2.0	3.0
MCP (21)	1.0	0.0
Feedlime	13.0	12.0
Salt	4.0	4.0
Vit/Min	3.0	3.0
Lucerne hay	160.0	75.0
Wheat/Maize straw	160.0	100.0

Table A.2 The ingredients for the low energy diet and the high energy diet used in phase 2.

Ingredients	Low Energy diet (As fed)	High Energy diet (As fed)
Hominy chop	130.0	60.0
Maize meal	170.0	495.0
Brewer's grain	200.0	120.0
Molasses	90.0	80.0
Urea (feed grade)	8.0	8.0
Cotton OCM (26)	40.0	40.0
Buffer (Sodiumbicarb)	1.0	3.0
MCP (21)	1.0	1.0
Feedlime	14.0	12.0
Salt	3.0	3.0
Vit/Min	3.0	3.0
Lucerne hay	60.0	75.0
Wheat/Maize straw	280.0	100.0

Addendum B: R script

```
################################################################################
#
#SALMON differential expression and annotation pipeline for well annotated genomes
#@author:charles.hefer@gmail.com
################################################################################
#
#Variables for the script below, this is customizable:
#the current working directory
Working directory <- "C:/Users/Ida Linde/Documents"
#experiment setup
experiment_file <- "samples.txt"
#in this case, it consists of a file with 5 colums:
#DIR -> the location of the .quant file (relative to working dir)
#SAMPLE -> Sample ID
#ANIMAL -> Animal ID
#BREED -> Breed
#DIET -> Diet
#Trancript to gene ID file:
transcript_gene <- "transcript_gene.tab"
#Import the required libraries
library("tximport")
library("tximportData")
library("readr")
library("DESeq2")
library("ggplot2")
library("vsn")
library("ggdendro")
library("reshape2")
library("pheatmap")
library("RColorBrewer")
#read from the samples directory
setwd(working_directory)
#list.files(getwd())
```

```
#This can be updated, consists of
#path, sample, id, breed
samples <- read.table(experiment_file, header=T)
files <- file.path(getwd(), "Salmon", samples$DIR, "quant.sf")
names <- samples$SAMPLE
names(files) <- paste0(names)
#this should return true, i.e all the quant files have been found:
all(file.exists(files))
#import the transcript ids
tx2gene <- read.table(transcript_gene, header=T)
#import the quant files, this takes some time
txi <- tximport(files, type="salmon", tx2gene=tx2gene)
#make sure everything is OK
names(txi)
head(txi$counts)
#head(txi$abundance)
#head(txi$length)
#Start DE analysis
sampleTable <- NULL
#Build the sample table from the experiment file, removing the dir column
sampleTable <- subset(samples, select = -DIR)
#Add rows to the sample table
rownames(sampleTable) <- colnames(txi$counts)
#ensure the rownames and the columns are the same, for sanity
sampleTable
#DE test for breed effect, controlling for diet
dds_breed <- DESeqDataSetFromTximport(txi, sampleTable, design=~DIET+BREED)
#test for diet effect, controlling for breed
dds_diet <- DESeqDataSetFromTximport(txi, sampleTable, ~BREED+DIET)
########################################
#Define Some functions to extract data from a correlation matrix
########################################
#extract the lower triangle
get_lower_tri<-function(cormat){
  cormat[upper.tri(cormat)] <- NA
```

```
  return(cormat)
}
#extract the upper triangle
get_upper_tri <- function(cormat){
  cormat[lower.tri(cormat)]<- NA
  return(cormat)
}
#Cluster the correlations
reorder_cormat <- function(cormat){
  # Use correlation between variables as distance
  dd <- as.dist((1-cormat)/2)
  hc <- hclust(dd)
  cormat <-cormat[hc$order, hc$order]
}
save_pheatmap_pdf <- function(x, filename, width=7, height=7) {
  stopifnot(!missing(x))
  stopifnot(!missing(filename))
  pdf(filename, width=width, height=height)
  grid::grid.newpage()
  grid::grid.draw(x$gtable)
  dev.off()
}
#differential expression analysis function below
differential_expression <- function(dds, name) {
  #for testing purposes
  #dds <- dds_diet
  #write the raw count data to a csv
  write.csv(assay(dds), file=paste0(name, "_raw_counts.tab"))
  #get rid of any zero values
  #make object smaller
  dds <- dds[ rowSums(counts(dds)) > 1, ]
  #get the number of reads per sample
  colSums(assay(dds))
    #perform the differential expression
  dds <- DESeq(dds)
```

```
  #make sure of the design
  design(dds)
  res <- results(dds)
    #description of the results column
  mcols(res, use.names=TRUE)
  #get a summary, not used
  summary(res)
  #sum(res_breed$padj < 0.1, na.rm=TRUE)
  #order the p-values
  resOrdered <- res[order(res$pvalue),]
  write.csv(as.data.frame(resOrdered), file=paste0(name, "_pvalues.tab"))
  #get the most upregulated and the most downregulated
  resSig <- subset(res, res$padj < 0.1)
  summary(resSig)
  write.csv(as.data.frame(resSig[ order( resSig$log2FoldChange ), ], 4), file=paste0(name,
"_significant_padj.tab"))
  jpeg(paste0(name, "_MA_Plot.jpeg"))
  MA_plot <- plotMA(res, ylim=c(2,2))
  dev.off()
  #dispersion plot
  jpeg(paste0(name, "_dispersion.jpeg"))
  plotDispEsts( dds, ylim = c(1e-6, 1e2) )
  dev.off()
  #convert the result to a data frame
  res_df <- as.data.frame(res)
  ggplot(res_df, aes(res$padj)) + geom_histogram() +
    labs(title="Adjusted P-value distribution") +
    labs(x="Adjusted p-value") +
    theme_bw()
  attr(res,"filterThreshold")
  #log transform the count data
  rld <- rlog(dds, blind=FALSE)
  head(assay(rld), 3)
  sampleDists <- dist(t(assay(rld))
  sampleDistMatrix <- as.matrix(sampleDists)
```

```
rownames(sampleDistMatrix) <- paste(rld$BREED, rld$SAMPLE, sep="-")
colnames(sampleDistMatrix) <- paste(rld$BREED, rld$SAMPLE, sep="-")
colors <- colorRampPalette( rev(brewer.pal(9, "Blues")) )(255)
distance_heatmap <- pheatmap(sampleDistMatrix,
         clustering_distance_rows=sampleDists,
         clustering_distance_cols=sampleDists,
         col=colors)
save_pheatmap_pdf(distance_heatmap, paste0(name,"_distance_heatmap.pdf"))
#Correlation plot of the samples using the rlog transformed data
cormat <- round(cor(assay(rld)), 2)
#Cluster the correlation matrix
cormat_reordered <- reorder_cormat(cormat)
#extract the upper triangle
upper_tri <- get_upper_tri(cormat_reordered)
#melt the correlation matrix for plotting
melted_cormat <- melt(upper_tri, na.rm=TRUE)
ggheatmap_red <- ggplot(melted_cormat, aes(Var2, Var1, fill = value))+
  geom_tile(color = "white")+
  scale_fill_gradient2(low = "blue", high = "red", mid = "white",
                midpoint = 0, limit = c(-1,1), space = "Lab",
                name="Pearson\nCorrelation") +
  theme_minimal()+ # minimal theme
  theme(axis.text.x = element_text(angle = 90, vjust = 1,
                           size = 12, hjust = 1))+
  coord_fixed() +
  geom_text(aes(Var2, Var1, label = value), color = "black", size = 4) +
  theme(
    axis.title.x = element_blank(),
    axis.title.y = element_blank(),
    panel.grid.major = element_blank(),
    panel.border = element_blank(),
    panel.background = element_blank(),
    axis.ticks = element_blank(),
    legend.justification = c(1, 0),
    legend.position = c(0.6, 0.7),
```

```
    legend.direction = "horizontal")+
  guides(fill = guide_colorbar(barwidth = 7, barheight = 1,
                               title.position = "top", title.hjust = 0.5))
 jpeg(paste0(name, "_SD_sample_correlation.jpeg"))
 ggheatmap_red
 dev.off()
 #dendogram based on the rld distance correlation matrix
 jpeg(paste0(name, "_dendrogram.jpeg"))
 hc <- hclust(dist(cormat_reordered), "ave")
 ggdendrogram(hc, rotate = FALSE, size = 2)
 dev.off()
 #heatmap of most expressed genes
 select <- order(rowMeans(counts(dds,normalized=TRUE)),
                 decreasing=TRUE)[1:50]
 df <- as.data.frame(colData(dds)[,c("BREED", "DIET")])
 gene_expr_heatmap <- pheatmap(assay(rld)[select,], cluster_rows=FALSE,
show_rownames=TRUE,
          cluster_cols=FALSE, annotation_col=df)
save_pheatmap_pdf(gene_expr_heatmap, paste0(name,"_gene_heatmap.pdf"))
 plotPCA(rld, intgroup=c("SAMPLE", "BREED"))
}
differential_expression(dds_breed, "breed_controlling_for_diet")
differential_expression(dds_diet, "diet_controlling_for_breed")
```

Addendum C: Differentially expressed genes between the treatment groups

Table C.1 the differentially expressed genes between the Bonsmara's that received the high energy diet and the Bonsmara's that received the low energy diet.

Gene name	Description	*log2FoldChange	Adjusted p-value
ACSL6	Long-chain-fatty-acid--CoA ligase 6 Acyl-CoA synthetase long-chain family member 6	-0.61	0.0793
ACTL8	Actin-like protein 8	-1.18	0.0349
ADAMTS8	A disintegrin and metalloproteinase with thrombospondin motifs 8	-2.19	3.2442e-05
AKR1C3	Aldo-keto reductase family 1 member C1	2.48	0.0024
ANKRD9	Ankyrin repeat domain-containing protein 9	-0.75	0.0091

AVP	Vasopressin-neurophysin 2-copeptin	-4.53	0.0010
CALM	Calmodulin, striated muscle	-0.38	0.0019
CALM3	Calmodulin	0.69	0.0192
CAPN11	Calpain-11	-0.55	0.0509
CASP8	Caspase-8	1.06	0.0001
CENPP	Centromere protein P	2.06	0.0001
CHRNE	Acetylcholine receptor subunit epsilon	0.39	0.0480
CLRN2	Clarin-2	5.02	6.7583e-05
CRYBB1	Beta-crystallin B1	-1.09	0.0023
CYB561D1	Cytochrome b561 domain-containing protein 1	-1.13	0.0005
CYP3A5	Cytochrome P450 3A4	-1.51	0.0938
DYNC1I1	Cytoplasmic dynein 1 intermediate chain 1	2.58	0.0208
ELMOD1	ELMO domain-containing protein 1	-2.92	0.0378
FAM131B	Protein FAM131B	-3.31	0.0002
FBP1	Fructose-1,6-bisphosphatase 1	-2.11	0.0166
FOXO1	Forkhead box protein O1	1.45	0.0033
GDF1	Embryonic growth/differentiation factor 1	-1.12	0.0017
GSTA2	Glutathione S-transferase A3	4.54	0.0006
HES1	Transcription factor HES-1	-1.06	0.0071
HES4	Transcription factor HES-4	1.48	0.0986
HES6	Transcription cofactor HES-6	-1.18	0.0009
HSPA6	Heat shock 70 kDa protein 6-related	2.21	0.0589
IRS2	Insulin receptor substrate 2	1.20	0.0401
LGR6	Leucine-rich repeat-containing G-protein coupled receptor 6	-2.21	0.0108
LOC100141215	MCG22096	-2.26	0.0206
MYCT1	Myc target protein 1	1.07	0.0388
MYH8	Myosin-8	2.29	0.0008
MYO1A	Unconventional myosin-Ia	-1.25	0.0241
NPAS4	Neuronal PAS domain-containing protein 4	-2.65	0.0046
NR4A3	Nuclear receptor subfamily 4 group A member 3	3.13	0.0048
NT5DC3	5'-nucleotidase domain-containing protein 3	2.06	0.0001

OXT	Oxytocin-neurophysin 1	-7.66	0.0009
POMC	Pro-opiomelanocortin	-0.64	0.0044
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	1.96	0.0103
PRKAG2	5'-AMP-activated protein kinase subunit gamma-2	1.17	1.1178e-05
PVALB	Parvalbumin alpha	-2.01	0.0410
RSAD2	Radical S-adenosyl methionine domain-containing protein 2	2.13	0.0005
RXFP2	Relaxin receptor 2	1.62	3.2016e-05
SIX2	Homeobox protein SIX2	-1.05	0.0095
SLC22A14	Solute carrier family 22 member 14	-2.05	0.0042
SLC25A33	Solute carrier family 25 member 33	2.13	3.8373e-06
SLC36A4	Proton-coupled amino acid transporter 4	-3.22	0.0003
SNAI3	Zinc finger protein SNAI3	2.79	3.8902e-06
SPTA1	Spectrin alpha chain, erythrocytic 1	2.03	0.0023
STAB2	Stabilin-2	2.48	0.0022
SYNJ2	Synaptojanin-2	2.81	0.0011
TMEM102	Transmembrane protein 102	-2.17	0.0118
TNFSF10	Tumor necrosis factor ligand superfamily member 10	2.08	9.7032e-07
TUBB3	Tubulin beta-3 chain	2.91	0.0198
unassigned	Cytochrome c oxidase subunit 6B1	3.05	0.0003
unassigned	Histone H3.1	-3.87	0.0271
unassigned	Glycine cleavage system H protein, mitochondrial	-6.56	0.0971
unassigned	60S ribosomal protein L35	8.22	0.0011

*Positive log2fold change indicates highly expressed in the bulls that received the low energy diet
Negative log2fold change indicates highly expressed in the bulls that received the high energy diet

Table C.2 The differential expressed genes between the Nguni's that received the low energy diet and the Nguni's that received the high energy diet.

Gene name	Family	*log2 FoldChange	Adjusted p-value

ABCC2	Canalicular multispecific organic anion transporter 1	-4.05	0.0207
ACTC1	Actin, alpha cardiac muscle 1	-1.29	0.0082
ADCY10	SUBFAMILY NOT NAMED	1.21	0.0935
ADCY8	Adenylate cyclase type 8	2.58	0.0826
AGAP1	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 1	-1.23	0.0552
ARL10	ADP-ribosylation factor-like protein 10	2.05	0.0242
ATOH8	Protein atonal homolog 8	1.14	0.0148
B3GAT1	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1	-1.02	0.0328
C28H10ORF116	Adipogenesis regulatory factor Chromosome 10 open reading frame 116 ortholog	1.23	0.0794
C5AR1	C5a anaphylatoxin chemotactic receptor 1	3.02	0.0027
CACNA1E	Voltage-dependent R-type calcium channel subunit alpha-1E	-1.66	0.0789
CALY	Neuron-specific vesicular protein calcyon	3.13	0.0982
CBLN4	Cerebellin-4	-1.30	0.0688
CCL19	C-C motif chemokine 19	1.25	0.0609
CCL21	C-C motif chemokine 21	1.71	0.0032
CEP85L	Centrosomal protein of 85 kDa-like	1.77	0.0503
CHD5	Chromodomain-helicase-DNA-binding protein 5	2.37	0.0129
CHRND	Acetylcholine receptor subunit delta	-2.58	6.0848e-05
COMMD8	COMM domain-containing protein 8	-1.62	0.0956
COX1	Cytochrome c oxidase subunit 1	0.90	0.0688
DIP2C	Disco-interacting protein 2 homolog C	-1.28	0.0376
DPF1	Zinc finger protein neuro-d4	1.30	0.0242
DPYSL4	Dihydropyrimidinase-related protein 4	1.73	0.0689
FAM69C	Protein FAM69C	1.52	0.0634
FHL1	Four and a half LIM domains protein 1	-1.12	0.0487
FSD2	Fibronectin type III and SPRY domain-containing protein 2	-1.02	0.0473

GADD45G	Growth arrest and DNA damage-inducible protein GADD45 gamma	1.96	0.0057
GALNT14	Polypeptide N-acetylgalactosaminyltransferase 14	1.10	0.0987
GAN	Gigaxonin	1.06	0.0808
GRB7	Growth factor receptor-bound protein 7	2.62	0.0957
GREB1	Protein GREB1	-1.61	0.0623
GSR	Glutathione reductase, mitochondrial	-1.27	0.0798
GSTA3	Glutathione S-transferase A3	7.47	0.0019
GTPBP10	GTP-binding protein 10	-0.95	0.0982
GYG1	Glycogenin-1	-1.08	0.0969
HES2	Transcription factor HES-2	1.10	0.0013
HIF1AN	Hypoxia-inducible factor 1-alpha inhibitor	-1.12	0.0770
HIPK2	Homeodomain-interacting protein kinase 2	-1.50	0.0770
IKZF4	Zinc finger protein Eos	-1.38	0.0610
ITPRIPL2	Inositol 1,4,5-trisphosphate receptor-interacting protein-like 2	-2.11	0.0808
JSP.1	Class Ib MHC antigen Qa-2-related	1.46	0.0366
KCNC4	Potassium voltage-gated channel subfamily C member 4	1.26	0.0260
KCNQ5	Potassium voltage-gated channel subfamily KQT member 5	-1.36	0.0761
LAD1	Ladinin-1	1.85	0.0721
LOC101906927	Histone H2B	1.46	0.0306
LOC407241	Krueppel-like factor 15	1.01	0.0113
LOC786095	NKG2D ligand 4	-1.41	0.0721
MAP1B	Microtubule-associated protein 1B	1.42	0.0620
MAPK6	Mitogen-activated protein kinase 6	-1.43	0.0716
MCHR1	Melanin-concentrating hormone receptor 1	-1.71	0.0276
MT3	Metallothionein-3	1.37	0.0126
MYPN	Myopalladin	-1.01	0.0448
NADK2	NAD kinase 2, mitochondrial	-1.25	0.0610
NAPSA	Napsin-A	1.11	0.0646
NAT11	N-alpha-acetyltransferase 40	-1.35	0.0892
PAX8	Paired box protein Pax-8	-2.29	0.0610

PDZRN3	E3 ubiquitin-protein ligase PDZRN3	-1.19	0.0304
PITX2	Pituitary homeobox 2	-1.09	0.0003
PPP1R3A	Protein phosphatase 1 regulatory subunit 3A	-1.00	0.0808
PPP1R3B	Protein phosphatase 1 regulatory subunit 3B	-1.51	0.0808
PRSS23	Serine protease 23	-1.23	0.0009
PSMC3IP	Homologous-pairing protein 2 homolog	1.36	0.0421
RAB40B	Ras-related protein Rab-40B	-1.46	0.0025
RELT	Tumor necrosis factor receptor superfamily member 19L	1.32	0.0376
SCAF8	Protein SCAF8	-1.14	0.0525
SHISA2	Protein shisa-2 homolog	-1.22	0.0643
SLC38A5	Sodium-coupled neutral amino acid transporter 5	1.22	0.0982
SLC41A3	Solute carrier family 41 member 3	1.19	0.0721
SLC5A6	Sodium-dependent multivitamin transporter	1.66	1.6004e-05
SMPD2	Sphingomyelin phosphodiesterase 2	1.16	0.0032
TCN1	Transcobalamin-1	1.53	0.0721
TRDN	Triadin	1.36	0.0458
TUBB3	Tubulin beta-3 chain	-5.23	0.0041
UBD	Ubiquitin D	-1.11	0.0617
unassigned	Cystatin-M	1.45	0.0458
unassigned	Cystatin-M	1.51	0.0761
unassigned	Cyclin-H	1.52	0.0990
unassigned		-1.66	0.0383
unassigned	Mitotic spindle assembly checkpoint protein MAD2A	-3.29	0.0770
unassigned	Protein Gm21964	3.75	3.7814e-17
unassigned	Glycine cleavage system H protein, mitochondrial	-6.80	0.0768
USP28	Ubiquitin carboxyl-terminal hydrolase 28	-1.02	0.0006
ZBED6	Zinc finger BED domain-containing protein 6	1.83	0.0175
ZNF211	Zinc finger protein 211	-1.28	0.0503

*Positive log2fold change indicates highly expressed in the bulls that received the low energy diet

Negative log2fold change indicates highly expressed in the bulls that received the high energy diet

Table C.3 The differentially expressed genes between the Bonsmara's that received the high energy diet and the Nguni's that received the high energy diet.

Gene name	Family/Subfamily	Log 2 Fold change	Adjusted p-value
ABCC2	Canalicular multispecific organic anion transporter 1	1.90	0.0917
ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	1.52	2.9836e-05
ACBD7	Acyl-CoA-binding domain-containing protein 7	2.72	3.9501e-11
ADAMTS8	A disintegrin and metalloproteinase with thrombospondin motifs 8	-2.20	0.0039
ADIPOQ	Adiponectin	-1.84	0.0064
AK7	Adenylate kinase 7	1.43	0.0807
AKR1C3	Aldo-keto reductase family 1 member C1	2.76	0.0038
ALDH1A1	Retinal dehydrogenase 1	1.78	5.3396e-08
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein	-1.87	0.0081
ANGPTL4	Angiopoietin-related protein 4	1.62	0.0586
ANKRD52	Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit C	1.58	2.6700e-06
ARHGAP36	Rho GTPase-activating protein 36	2.10	0.0004
ASB1	Ankyrin repeat and SOCS box protein 1	1.81	0.0404
ASIP	Agouti-signaling protein	-2.62	0.0900
ATCAY	Caytaxin	-1.53	0.0057
ATXN7L2	Ataxin-7-like protein 2	-1.84	1.2290e-12
AVP	Vasopressin-neurophysin 2-copeptin	-4.16	0.0031
BEGAIN	Brain-enriched guanylate kinase-associated protein	-1.59	0.0346

BRWD3	Bromodomain and WD repeat-containing protein 3	-1.35	0.0088
C18orf54	Lung adenoma susceptibility protein 2	1.41	0.0924
C1QA	Complement C1q subcomponent subunit A	-1.39	0.0003
C2CD4A	C2 calcium-dependent domain-containing protein 4A	-1.61	0.0586
CACNA1E	Voltage-dependent R-type calcium channel subunit alpha-1E	2.15	0.0005
CALM2	SUBFAMILY NOT NAMED	1.14	0.0268
CALM3	Calmodulin	0.81	0.0171
CAPN11	Calpain-11	-0.98	0.0009
Cationic trypsin;unassigned	Protein 2210010C04Rik	3.70	0.0240
CCL17	C-C motif chemokine 17	-1.60	0.0644
CCL24	C-C motif chemokine 24	-1.35	0.0066
CDC14B	Dual specificity protein phosphatase CDC14B-related	1.40	0.0288
CECR1	Adenosine deaminase CECR1	-1.67	0.0003
CENPP	Centromere protein P	1.54	0.0354
CHAC1	Glutathione-specific gamma-glutamylcyclotransferase 1	-1.56	0.0362
CHADL	Chondroadherin-like protein	-1.83	0.0166
CHRND	Acetylcholine receptor subunit delta	2.83	8.9719e-08
CHST13	Carbohydrate sulfotransferase 13	-1.80	0.0611
CIDEC	Cell death activator CIDE-3	-1.83	0.0200
CLEC11A	C-type lectin domain family 11 member A	-1.46	5.7251e-05
CLRN2	Clarin-2	3.94	0.0597
COL13A1	Collagen alpha-1(XIII) chain	-1.34	0.0353
COL22A1	Collagen alpha-1(XXII) chain	-1.32	0.0983
COX3	Cytochrome c oxidase subunit 3	-1.32	0.0005
COX7C	Cytochrome c oxidase subunit 7C, mitochondrial	1.95	6.2020e-05
CRAMP1	Protein cramped-like	2.66	0.0030
CRB1	Protein crumbs homolog 1	-1.72	0.0022
CYP2W1	Cytochrome P450 2W1	-1.47	0.0049

CYP3A5	Cytochrome P450 3A4 -Cytochrome P450, family 3, subfamily A, polypeptide 5	-1.53	0.0390
Cystatin	Cystatin-M	-1.46	0.0013
DCK	Deoxycytidine kinase	1.47	0.0551
DOCK3	Dedicator of cytokinesis protein 3	1.58	0.0992
DOK2	Docking protein 2	-1.52	0.0054
DYNC1I1	Cytoplasmic dynein 1 intermediate chain 1	2.41	2.9731e-07
ELMOD1	ELMO domain-containing protein 1	-4.68	4.00e-07
ESPNL	Espin-like protein	-1.33	0.0143
FAIM2	Protein lifeguard 2	2.43	0.0077
FAM131B	Protein FAM131B	-3.00	1.0052e-05
FBXO45	F-box/SPRY domain-containing protein 1	2.32	0.0586
FNDC3B	Fibronectin type III domain-containing protein 3B	1.74	0.0160
FOS	Proto-oncogene c-Fos	-1.74	0.0433
FOXJ3	Forkhead box protein J3	1.45	0.0484
FRRS1L	DOMON domain-containing protein FRRS1L	1.95	0.0156
GDF11	Growth/differentiation factor 11	1.44	0.0777
GLCCI1	Glucocorticoid-induced transcript 1 protein	2.60	0.0076
GPR142	G-protein coupled receptor 142-related	3.98	9.8740e-05
GPR37L1	Prosaposin receptor GPR37L1	-1.27	0.0399
GPT2	Alanine aminotransferase 2	1.29	0.0165
GPX2	Glutathione peroxidase 2	1.58	0.0232
GREB1	Protein GREB1	-1.23	0.0608
GRID1	Glutamate receptor ionotropic, delta-1	-1.51	0.0056
GSK3B	Glycogen synthase kinase-3 beta	1.46	0.0051
GSKIP	GSK3-beta interaction protein	1.51	7.8739e-05
GSR	Glutathione reductase, mitochondrial	1.18	0.0047
GSTA3	Glutathione S-transferase A3	-8.89	2.7271e-06
GSTM2	Glutathione S-transferase Mu 4	-1.76	0.0004
HECA	Headcase protein homolog	1.69	0.0771
HIG2	Hypoxia-inducible lipid droplet-associated protein	1.46	0.0443
HIPK2	Homeodomain-interacting protein kinase 2	1.49	0.0122
HSPA6	Heat shock 70 kDa protein 6-related	1.51	0.0631

ICOSLG	ICOS ligand	-1.43	0.0124
IKZF4	Zinc finger protein Eos	1.48	0.0034
INSIG2	Insulin-induced gene 2 protein	0.98	0.0085
IQCA1	IQ and AAA domain-containing protein 1	-1.92	0.0004
IQUB	IQ and ubiquitin-like domain-containing protein	1.47	0.0765
IRS1	Insulin receptor substrate 1	1.05	0.0260
IYD	Iodotyrosine deiodinase 1	1.52	0.0129
JSP.1	Class Ib MHC antigen Qa-2-related	-1.51	1.1119e-05
KBTBD7	Kelch repeat and BTB domain-containing protein 7	1.62	0.0337
KCNE1L	Potassium voltage-gated channel subfamily E regulatory beta subunit 5	1.40	0.0338
KDM1B	Lysine-specific histone demethylase 1B	1.32	0.0075
KIAA0513	RH29724p	1.54	0.0347
KIF25	Kinesin-like protein KIF25	-1.54	0.0020
KLF12	Krueppel-like factor 12	2.01	0.0118
KLHL11	Kelch-like protein 11	2.15	0.0443
KLRF2	Killer cell lectin-like receptor subfamily F member 2	-4.33	0.0063
KRAS	GTPase KRas	1.55	0.0164
LACTB2	Beta-lactamase-like protein 2	1.39	0.0066
LAD1	Ladinin-1	-1.59	0.0042
LAG3	Lymphocyte activation gene 3 protein	-1.42	0.0130
LARP4	La-related protein 4	1.52	3.6808e-06
LCORL	Ligand-dependent nuclear receptor corepressor-like protein	1.54	0.0630
LMBR1L	Protein LMBR1L	-1.63	9.1836e-09
LOC104968632	Transmembrane protein 33	1.42	0.0328
LOC782884	Aldo-keto reductase family 1 member C1	1.55	0.0092
LRRC27	Leucine-rich repeat-containing protein 27	1.41	0.0657
LTF	Lactotransferrin	-1.62	0.0586
MAP1B	Microtubule-associated protein 1B	-1.54	0.0033
MCHR1	Melanin-concentrating hormone receptor 1	2.01	0.0002

MEGF6	Multiple epidermal growth factor-like domains protein 6	-1.56	0.0284
MMP11	Stromelysin-3	-1.13	0.0276
MOGAT1	2-acylglycerol O-acyltransferase 1	-4.46	1.2778e-07
MRPL15	39S ribosomal protein L15, mitochondrial	1.30	4.1787e-08
MT-ATP6	ATP synthase subunit a	-1.13	0.0045
MT-ND4L	NADH-ubiquinone oxidoreductase chain 4L	-1.50	0.0022
MT-ND6	NADH-ubiquinone oxidoreductase chain 6	-1.34	9.1407e-05
MTX3	Metaxin-3	1.30	0.0631
MUC4	Mucin-4	1.75	0.0475
MYH8	Myosin-8	1.92	0.0021
ND1	NADH-ubiquinone oxidoreductase chain 1	-1.31	0.0011
ND5	NADH-ubiquinone oxidoreductase chain 5	-1.35	0.0006
NFAM1	NFAT activation molecule 1	-1.37	0.0432
NK2A;LOC104968634	Granulysin	-1.49	0.0042
NPAS4	Neuronal PAS domain-containing protein 4	-3.00	0.0009
NRIP1	Nuclear receptor-interacting protein 1	1.37	0.0959
NTNG2	Netrin-G2	-1.42	0.0198
OR51E1	Olfactory receptor 51E1	1.43	0.0236
OTOR	Melanoma inhibitory activity protein 3-related -Otoraplin	-1.31	0.0556
OVCH2	Ovochymase-2	-1.24	0.0185
OXT	Oxytocin-neurophysin 1	-3.90	0.0070
PAPLN	Papilin	-1.83	0.0486
PARP8	Poly [ADP-ribose] polymerase 8	-1.31	0.0058
PDCD1	Programmed cell death protein 1	-1.71	0.0075
PITX2	Pituitary homeobox 2	0.50	0.0140
PLTP	Phospholipid transfer protein	-1.65	4.1700e-06
POLQ	DNA polymerase theta	1.77	0.0180
POMC	Pro-opiomelanocortin	-0.61	0.0623
PPP2R3A	Serine/threonine-protein phosphatase 2A regulatory subunit B'' subunit alpha	1.39	0.0005
PRKAG2	5'-AMP-activated protein kinase subunit gamma-2	1.29	0.0191
PRL	Prolactin	-2.19	0.0066

RAB40B	Ras-related protein Rab-40B	1.37	0.0003
RAB6B	Ras-related protein Rab-6B	-2.83	3.6808e-06
RBP7	Retinoid-binding protein 7	1.70	0.0467
RCOR1	REST corepressor 1	1.39	0.0986
RFESD	Rieske domain-containing protein	1.35	0.0585
RFX2	DNA-binding protein RFX2	2.29	0.0019
Ribosomal protein;unassigned	60S ribosomal protein L10a	5.22	0.0353
RNASE1	Ribonuclease pancreatic	-1.74	0.0773
RXFP1	Relaxin receptor 1	2.90	0.0005
RYR2	Ryanodine receptor 2	-1.57	0.0485
S100A7	Protein S100-A7A	-1.33	0.0915
SAMD3	Sterile alpha motif domain-containing protein 3	-1.32	0.0829
SARDH	Sarcosine dehydrogenase, mitochondrial	-1.32	0.0096
SERINC5	Serine incorporator 5	1.53	0.0362
Serine/threonine-protein phosphatase	Serine/threonine-protein phosphatase 2B catalytic subunit beta isoform	1.48	0.0219
SERPINE1	Plasminogen activator inhibitor 1	-1.36	0.0101
SHISA3	Protein shisa-3 homolog	-1.43	0.0608
SIGLEC5	Sialic acid-binding Ig-like lectin 14-related	-1.40	0.0137
SIRT4	NAD-dependent protein lipoamidase sirtuin-4, mitochondrial	0.45	0.0534
SIRT5	NAD-dependent protein deacylase sirtuin-5, mitochondrial	0.46	0.0028
SLC22A23	Solute carrier family 22 member 23	-1.35	0.0001
SLC25A46	Solute carrier family 25 member 46	1.41	0.0414
SLC28A1	Sodium/nucleoside cotransporter 1	-2.81	1.7156e-07
SLC36A4	Proton-coupled amino acid transporter 4	-3.23	0.0034
SLC39A10	Zinc transporter ZIP10	1.48	0.0051
SLC39A8	Zinc transporter ZIP8	1.58	0.0012
SLC9A2	Sodium/hydrogen exchanger 2	1.52	0.0217
SMPD2	Sphingomyelin phosphodiesterase 2	-1.31	0.0001
SMPX	Small muscular protein	1.24	7.0060e-05
SORD	Sorbitol dehydrogenase	1.35	0.0040

SPTA1	Spectrin alpha chain, erythrocytic 1	1.66	0.0067
STK26	Serine/threonine-protein kinase 26	1.81	0.0024
STMN2	Stathmin-2	-2.03	5.6720e-05
STX7	Syntaxin-7	1.34	9.8070e-05
SYCP2L	Synaptonemal complex protein 2-like	-3.63	1.2074e-07
SYT5	Synaptotagmin-5	-1.37	0.0253
TAB3	TGF-beta-activated kinase 1 and MAP3K7-binding protein 3	1.87	0.0594
TEX9	Testis-expressed sequence 9 protein	1.20	0.0140
TMED5	Transmembrane emp24 domain-containing protein 5	1.48	0.0034
TMEM198	Transmembrane protein 198	-1.97	0.0078
TUBB3	Tubulin beta-3 chain	4.19	0.0010
TUSC5	Tumor suppressor candidate 5	-1.83	0.0181
Tyrosine-protein kinase receptor	NT-3 growth factor receptor	-1.84	0.0014
unassigned	Purine nucleoside phosphorylase	1.51	0.0498
unassigned	E3 ubiquitin-protein ligase RNF213	1.59	0.0682
unassigned	Nucleolin	5.49	0.0051
XCL1	Cytokine SCM-1 beta-related -Lymphotactin	-2.18	0.0633
ZFR2	Zinc finger RNA-binding protein 2	-1.99	0.0169
ZMIZ1	Zinc finger MIZ domain-containing protein 1	1.55	0.0057
ZNF445	Zinc finger protein 445	2.02	0.0417

*Positive log2fold change indicates highly expressed in the Nguni

Negative log2fold change indicates highly expressed in the Bonsmara

Table C.4 The differentially expressed gene between the Bonsmara's that received the low energy diet and the Nguni's that received the low energy diet.

Gene name	**Family**	**log2FoldChange**	**padj**
ADCY8	Adenylate cyclase type 8	3.32	0.0201
ADIPOR2	Adiponectin receptor protein 2	0.67	0.0874
AKAP4	A-kinase anchor protein 4	1.60	0.0687
ANKRD9	Ankyrin repeat domain-containing protein 9	1.90	0.0027
ARHGAP15	Rho GTPase-activating protein 15	-2.23	0.0240

ARHGAP27	Rho GTPase-activating protein 27	-1.69	0.0061
ARL10	ADP-ribosylation factor-like protein 10	1.92	0.0072
ASIP	Agouti-signaling protein	-5.53	4.5113e-20
ATRX	Transcriptional regulator ATRX	1.70	0.0156
BBS10	Bardet-Biedl syndrome 10 protein	-2.79	0.0107
BHLHE40	Class E basic helix-loop-helix protein 40	-1.63	0.0033
BPIFA2A	BPI fold-containing family A member 2	4.69	0.0638
C6orf25	Protein G6b	2.05	0.0847
CALM	Calmodulin, striated muscle	0.52	0.0608
CALM2	Calmodulin	1.32	0.0264
CALY	Neuron-specific vesicular protein calcyon	2.19	0.0780
CASP8	Caspase-8	-1.62	0.0006
CCL21	C-C motif chemokine 21	1.64	0.0005
CCL8	C-C motif chemokine 8	-2.56	0.0844
CD200	OX-2 membrane glycoprotein	-2.28	8.3398e-05
CDK1	Cyclin-dependent kinase 1	-2.00	0.0654
CDKL2	Cyclin-dependent kinase-like 2	2.90	0.0128
CELSR1	Cadherin EGF LAG seven-pass G-type receptor 1	-2.71	0.0299
CENPP	Centromere protein P	-1.81	0.0659
CHD5	Chromodomain-helicase-DNA-binding protein 5	2.12	0.0377
COQ10B	Coenzyme Q-binding protein COQ10 homolog B, mitochondrial	1.80	0.0102
CRB1	Protein crumbs homolog 1	-1.85	0.0657
CYCS	Cytochrome c	1.73	0.0012
CYP1A1	Cytochrome P450 1A1	-1.35	0.0085
CYP2B6	Cytochrome P450 2B6	2.00	0.0550
CYP2W1	Cytochrome P450 2W1	-1.68	0.0507
DENND2A	DENN domain-containing protein 2A	1.82	2.4193e-08
DIRAS3	GTP-binding protein Di-Ras3	1.61	0.0542
DLG4	Disks large homolog 4	1.73	0.0171
DNAJB13	DnaJ homolog subfamily B member 13	2.94	0.0704
DOK3	Docking protein 3	2.26	0.0474
DPYSL4	Dihydropyrimidinase-related protein 4	1.80	0.0250

DSG4	Desmoglein-4	-2.10	0.0138
EIF2C3	Protein argonaute-3	2.31	0.0267
ENHO	Adropin	2.00	5.4641e-08
ENKUR	Enkurin	1.69	0.0316
EPDR1	Mammalian ependymin-related protein 1	-1.94	0.0139
ERN1	Serine/threonine-protein kinase/endoribonuclease IRE1	3.42	0.0696
EVI2A	Protein EVI2A Ecotropic viral integration site 2A	-1.76	0.0576
FAM111A	Protein FAM111A	2.04	0.0569
FAM124B	Protein FAM124B	-2.52	6.6542e-05
FAM166B	Protein FAM166B	2.03	0.0045
FAM26E	Protein FAM26E	-2.66	0.0036
FAM3B	Protein FAM3B	-2.58	0.0680
FNIP2	Folliculin-interacting protein 2	-2.56	9.3931e-06
FOXN2	Forkhead box protein N2	-1.60	0.0869
FRRS1L	DOMON domain-containing protein	2.41	0.0276
FTSJ2	rRNA methyltransferase 2, mitochondrial	1.74	1.6111e-05
GADD45G	Growth arrest and DNA damage-inducible protein	2.76	2.4923e-05
GALNT3	Polypeptide N-acetylgalactosaminyltransferase 3	-2.21	0.0513
GAS1	Growth arrest-specific protein 1	-2.05	0.0155
GAS7	Growth arrest-specific protein 7	1.52	0.0518
GAST	Gastrin	2.24	0.0423
GDA	Guanine deaminase	-1.47	0.0542
GIMAP8	GTPase IMAP family member 8	-2.44	0.0011
GREB1	Protein GREB1	-2.42	0.0005
GSTA2	Glutathione S-transferase A3	-3.99	0.0021
GZMK	Granzyme K	-2.20	0.0654
HABP2	Hyaluronan-binding protein 2	1.60	0.0036
IFI44	Interferon-induced protein 44	-1.73	0.0098
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1B	-2.59	0.0271

IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	-2.60	0.0461
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	-1.75	2.7637e-05
IGFBP3	Insulin-like growth factor-binding protein 3	-1.10	0.0854
IGLL1	SUBFAMILY NOT NAMED	1.91	4.2525e-05
IL17B	Interleukin-17B	2.31	0.0730
ISG15	Ubiquitin-like protein ISG15	-1.59	0.0232
ITK	Tyrosine-protein kinase ITK/TSK	-1.67	0.0044
KCNG2	Potassium voltage-gated channel subfamily G member 2	2.35	9.6905e-05
KCNN4	Intermediate conductance calcium-activated potassium channel protein 4	2.49	0.0493
KIF11	Kinesin-like protein KIF11	-1.78	0.0545
KIF18B	Kinesin-like protein KIF18B	-1.74	0.0242
KLK12	Kallikrein-12	-1.88	0.0430
KLRF2	Killer cell lectin-like receptor subfamily F member 2	-2.49	0.0034
KRT1	Keratin, type II cytoskeletal 1	3.68	0.0302
LGR6	Leucine-rich repeat-containing G-protein coupled receptor 6	2.68	0.0544
LMTK3	Serine/threonine-protein kinase	2.26	0.0150
LOC574091	Nucleoside-diphosphate kinase NBR-A	4.13	0.0003
LOC615587	SUBFAMILY NOT NAMED	2.55	0.0007
LOC617313	Granzyme H	-2.03	0.0910
LOC781439	Methyltransferase-like protein 7A	-2.11	0.0102
LRRC32	Leucine-rich repeat-containing protein 32	-1.96	0.0198
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	-1.75	0.0201
MCU	Calcium uniporter protein, mitochondrial	-1.70	0.0071
MEGF11	Multiple epidermal growth factor-like domains protein 11	2.63	0.0801
MEGF6	Multiple epidermal growth factor-like domains protein 6	-2.06	0.0787
METTL21C	Protein-lysine methyltransferase	-2.50	0.0005

MIS18BP1	Mis18-binding protein 1	-2.09	0.0181
MPPED2	Metallophosphoesterase	-1.99	0.0207
MUM1L1	PWWP domain-containing protein	-1.67	0.0916
MX1	Interferon-induced GTP-binding protein	-1.62	0.0021
MXRA5	Matrix-remodeling-associated protein 5	-1.85	0.0085
MYBPH	Myosin-binding protein H-related	1.50	1.441e-09
MYCT1	Myc target protein 1	-2.03	0.0014
MYO3A	Myosin-IIIa	1.89	0.0661
MZT2	Mitotic-spindle organizing protein 2A-related	1.67	0.0262
NAT11	N-alpha-acetyltransferase 40	-1.92	0.0024
NFASC	Neurofascin	1.79	0.0469
NR4A3	Nuclear receptor subfamily 4 group A member 3	-2.48	0.0202
OASL	2'-5'-oligoadenylate synthase-like protein	-3.55	0.0099
OTOS	Otospiralin	-5.82	9.4858e-05
PANK1	Pantothenate kinase 1	-1.61	0.0021
PCDHA13	Protocadherin alpha-13	-2.24	0.0033
PCDHB7	Protocadherin beta-7	-2.80	0.0162
PCGF6	Polycomb group RING finger protein 6	-1.84	0.0554
PCYT1A	Choline-phosphate cytidylyltransferase A	1.62	0.0316
PITX2	Pituitary homeobox 2	-0.59	0.0757
PLCG2	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2	1.60	0.0465
PLEKHA7	Pleckstrin homology domain-containing family A member 7	-1.91	0.0654
PLXNC1	Plexin-C1	-1.90	0.0158
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	-1.63	0.0019
PPP1R3B	Protein phosphatase 1 regulatory subunit 3B	-2.12	0.0021
PRF1	Perforin-1	-1.99	0.0289
PRKAG3	5'-AMP-activated protein kinase subunit gamma-3	0.45	0.0467
PRR32	Proline-rich protein 32	2.03	8.766e-05
PSD4	PH and SEC7 domain-containing protein 4	-1.61	0.0641
PSMC3IP	Homologous-pairing protein 2 homolog	1.76	0.0014

PVALB	Parvalbumin alpha	1.91	0.0153
RHBG	Ammonium transporter Rh type B	1.80	0.0302
RSAD2	Radical S-adenosyl methionine domain-containing protein 2	-2.16	0.0101
RTP4	Receptor-transporting protein 4	-2.09	0.0006
SENP5	Sentrin-specific protease 5	2.40	0.0049
SERPINF2	Alpha-2-antiplasmin	2.53	0.0654
SHISA2	Protein shisa-2 homolog	-1.62	0.0023
SIX4	Homeobox protein	-1.30	0.0829
SLC22A14	Solute carrier family 22 member 14	3.29	0.0069
SLCO4A1	Solute carrier organic anion transporter family member 4A1	1.61	0.0231
SMC2	Structural maintenance of chromosomes protein 2	-1.80	0.0889
SMIM24	Small integral membrane protein 24	2.19	0.0163
SNAI3	Zinc finger protein SNAI3	-3.59	8.5532e-07
SOX7	Transcription factor SOX-7	-1.83	0.0072
SP3	Transcription factor Sp3	-1.58	0.0079
SPI1	Transcription factor PU.1	1.58	0.0904
SRPK3	SRSF protein kinase 3	0.73	0.0009
STAB2	Stabilin-2	-2.79	0.0131
SYCE1L	Synaptonemal complex central element protein 1-like	1.69	0.0581
SYNJ2	Synaptojanin-2	-2.19	0.0034
TARSL2	threonine--tRNA ligase 2, cytoplasmic-related	1.66	0.0278
TENM1	Teneurin-1	1.97	0.0296
THEMIS2	Protein THEMIS2	-1.74	0.0728
TNFRSF1B	Tumor necrosis factor receptor superfamily member 1B	-1.65	0.0177
TNFSF4	Tumor necrosis factor ligand superfamily member 4	-1.72	0.0511
TNNI3	Troponin I, cardiac muscle	1.76	0.0053
TNNT1	Troponin T, slow skeletal muscle	0.75	0.0575
TRHR	Thyrotropin-releasing hormone receptor	-2.71	0.0617
TUBB3	Tubulin beta-3 chain	-3.95	0.0102

UBD	Ubiquitin D	-1.91	9.1473e-06
unassigned	Cystatin-M	2.20	8.3680e-06
unassigned	60S ribosomal protein L35	-6.40	0.0142
WFIKKN1	WAP, Kazal, immunoglobulin, Kunitz and NTR domain-containing protein 1	3.57	0.0200
ZBED6	Zinc finger BED domain-containing protein 6	2.12	0.0060
ZBP1	Z-DNA-binding protein 1	-1.63	0.0158
ZNF623	Zinc finger protein 623	-1.75	0.0999
ZNF689	Zinc finger protein 689	-1.93	0.0192
ZNF710	Zinc finger protein 710	-1.68	0.0466

*Positive log2fold change indicates highly expressed in the Nguni

Negative log2fold change indicates highly expressed in the Bonsmara