COMPARATIVE MOLECULAR CHARACTERISATION OF ADIPOSE TISSUE

CHOONG SIEW SHEAN, DVM, MVSc.

Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

JULY 2016

Abstract

Adiposity is linked to reproductive efficiency as adipose tissue (AT) is known to influence the hypothalamic-pituitary-gonadal axis, although the exact roles of AT remain poorly understood. Different cattle and pig breeds exhibit different adiposity and reproductive characteristics. The hypothesis is that differences in adiposity underpin the phenotypic differences in reproductive performance in these animals. To test this hypothesis, next generation RNA sequencing analysis was completed of AT from (i) cattle (Holstein, Hereford and Aberdeen Angus cross breeds enrolled in a dietary protein feed trial), (ii) European Large White and Asian Meishan pigs and (iii) African elephants. Differentially expressed genes (DEG) were determined in each species and validation completed utilising quantitative reverse transcriptase PCR (qRT-PCR). Results revealed significant DEG involved in adipogenesis and lipogenesis between (i) breeds, (ii) resulting from the dietary protein intervention and (iii) sexes and age groups. Fatty acid (FA) composition of AT from different cattle breeds and dietary protein levels was determined using gas chromatography and compared, although significant difference was not observed. Gene networks related to vitamins A and D suggest potential nutritional influences on adipogenesis and lipogenesis, consequently vitamin levels in feed may impact adiposity and thereby animal reproductive performance. Results from the elephants indicated disparity in energy storage and utilisation between sexes and age groups, thus this can provide baseline information to improve captive population management of these animals in different life stages. By exploiting comparative inferences from previous

human and animal studies to the results obtained from this study, a positive influence of adiposity on fertility was identified. Future work should verify whether differential mRNA expression of genes of interest identified are reflected in protein expression. Also additional studies into the FA profiles of other biologically relevant tissues are warranted to enable comparison of FA uptake and usage between AT and other tissues.

Acknowledgement

First and foremost, I like to express my utmost gratitude to my supervisors, Professor Richard D. Emes, Dr Nigel P. Mongan, and Dr Lisa Yon for the opportunity to work on this project. Their relentless guidance, advice and support throughout the study has been incredible and I am eternally grateful. They are all accomplished in their respective fields, it was an extraordinary educational experience to be working with them, and I was much privileged to have such an opportunity.

I am also tremendously grateful of the help from Dr Viv Perry and Dr Alison Mostyn in obtaining the cattle and pig adipose samples respectively for this study, as well as their valuable advice and constructive suggestions. Mr John Schoefield from Dovecote Park Ltd was remarkably helpful and accommodating throughout the sample collection period, for that I am truly indebted.

I would also like to thank Dr Richard Talbot, Dr Pablo Fuentes-Utrillia, and Mrs Chieko Kontani from Edinburgh Genomics for the completion of next-generation sequencing and their valuable inputs on the samples and protocols.

This study would not be completed without the help and guidance from Dr Emeli Nilsson, Dr Hernan Pablo Fainberg, and Dr Dongfang Li in various aspects of laboratory works, and I am greatly appreciative of their relentless efforts.

To my laboratory group members, Miss Jennifer Edwards and Miss Siobhan Simpson, with whom I had countless stimulating discussions and exchange of ideas that were thought-provoking and inspirational, I would like to express my gratitude and to wish them the best of luck in their future ventures.

A special thanks to friends and colleagues with whom I have ventured through triumphs and failures during this past three years, for their help and support in and out of work. They have been wonderful and made this journey much more enjoyable and productive. They have definitely earned a special place in my heart.

Last but not least, to my mother, brother and sister, who have been very loving, understanding, patient, and supportive throughout the period of my study.

Dedication

I would like to dedicate this work to my late father, who had always believed in me and supportive of my choices in life.

Table of Contents

List of Tables

List of Figures

Abbreviations

AA	Aberdeen Angus
AAX	Aberdeen Angus crosses
ACACA	Acetyl-CoA carboxylase
ACTB	Actin β
ALA	α-linolenic acid
ASS1	Argininosuccinate synthase 1
AT	Adipose tissue
ATGL	Adipocyte triglyceride lipase
ATRA	All-*trans* retinoic acid
BAT	Brown adipose tissue
BEFS	Bovine embryonic fibroblasts
BCS	Body condition score
BSE	Bovine spongiform encephalitis
bTB	Bovine tuberculosis
C	Cytosine
C/EBP	CCAAT/enhancer binding protein
CA2	Carbonic anhydrase
ChIP-PET	Chromatin immunoprecipitation assays
CPT1	Carnitine palmitoyltransferase I
CRABP2	Cellular retinoic acid binding protein 2
Ct	Cycle threshold
DAG	Directed Acyclic Graphs
DE	Differential expression
DEG	Differentially expressed genes
DHA	Docosahexaenoic acid
E_2	17β-Estradiol
EBF1	Early B-cell factor 1
EFA	Essential fatty acid
ELOVL5	ELOVL fatty acid elongase 5
ELOVL6	ELOVL fatty acid elongase 6
EPA	Eicosapentaenoic acid
FA	Fatty acid
FASN	Fatty acid synthase
FC	Fold change
FFA	Free fatty acid
FMD	Food and mouth disease
FSH	Follicle-stimulating hormone
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GCs	Granulosa cells
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GR	Glucocorticoid receptor
HP	Adipose sample from high dietary protein group
HPG	Hypothalamic-pituitary-gonadal

HSL	Hormone sensitive lipase
hTB	Human tuberculosis
ICBF	Irish Cattle Breeding Federation
IGF-1	Insulin-like growth factor 1
IGFBP5	Insulin-like growth factor binding protein 5
IMF	Intramuscular fat
INSIG1	Insulin induced gene 1
IRF4	Interferon regulatory factor 4
IVF	*in-vitro* fertilisation
IVM	*in-vitro* maturation
L	Landrace
LA	Linoleic acid
LDLR	Low density lipoprotein receptor
LEP	Leptin
LEPR	Leptin receptor
LGALS12	Lectin, galactose binding, soluble 12
LH	Luteinizing hormone
LNA	Linolenic acid
LP	Adipose sample from low dietary protein group
LPL	Lipoprotein lipase
LW	Large White
LWxL	Large White X Landrace
mESCs	Mouse embryonic stem cells
MS	Meishan
MSCs	Mesenchymal stem cells
MUFA	Monounsaturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
NGS	Next generation sequencing
NNAT	Neuronatin
NPN	Non-protein nitrogen
OCFA	Odd chain fatty acid
PA	Pentadecanoic acid
PC	Pyruvate carboxylase
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PER2	Period homolog 2
PPARGC1	Peroxisome proliferator activated receptor γ coactivator 1
PPARγ	Peroxisome proliferator-activated receptor γ
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative reverse transcription PCR
RA	Retinoic acid
RAN	RAN, member RAS oncogene family
RAR	Retinoid acid receptor
RDP	Rumen degradable protein
RNAi	RNA interference

RIA	Radioimmunoassay
RNA-seq	RNA sequencing
RT	Reverse transcription
RUP	Rumen undegradable protein
RXRα	Retinoid X receptor α
SCD	Stearoyl-CoA desaturase
SCFA	Short chain fatty acid
SFA	Saturated fatty acid
SREBP1	Sterol regulatory element binding protein 1
TBP	TATA box binding protein
TCA	Tricarboxylic acid
TECs	Theca externa cells
TICs	Theca interna cells
TNF-α	Tumour necrosis factor-α
UCP1	Uncoupling protein 1
UCP2	Uncoupling protein 2
UXT	Ubiquitously-expressed, prefoldin-like chaperone
VDR	Vitamin D receptor
VLDL	Very low density lipoproteins
WAT	White adipose tissue
ZDF	Zucker diabetic fatty
ZLC	Zucker lean control

Chapter 1 General Introduction

1.1 Background

Comparing and contrasting between organisms is an ancient concept that likely dates back to the time human civilisation was established. The earliest record from ancient Greece showed that Aristotle (384 – 322 BC) utilised common characteristics of organisms to establish a classification system (Sanford et al., 2002). In modern times, comparative studies form the basis of phylogenetic classification, which describes the evolutionary processes that organisms have undergone, through comparison of phenotypic and functional features, especially with reference to adaptation to environmental changes (Garland et al., 2005). Today, comparative approaches have expanded beyond evolutionary biology and are often used to identify common and divergence in physiological traits and functional mechanisms across species. Such studies have informed research into the discovery of novel disease treatments, improvements in agricultural production, and yield of crops and livestock (Carlson, 2012).

The enormous potential of comparative studies lies in their capacity to infer knowledge learned from one species onto another. This is often necessary as restriction in terms of cost and space, ethical and welfare considerations, and short progeny generation time, such as in the case of using mice models for various research in human diseases, makes comparative studies an essential tool in biomedical research (Sanford et al., 2002, Melina, 2010, Carlson, 2012). Pigs and cows are two common domestic animals used, albeit infrequently, in

comparative research. Pigs have served as a preferred model for biomedical research because of their genetic and physiological similarities to humans, in terms of organ sizes and being monogastric and omnivorous (Wernersson et al., 2005, Walters and Prather, 2013) in renal diseases, gastrointestinal microbiome, type II diabetes, and animal-to-human xenotransplantation (Magyar et al., 1966, Greene et al., 1983, Vodicka et al., 2005, Zhang et al., 2013, Dunford et al., 2014). Cattle are natural hosts for infectious diseases that affect people such as tuberculosis, salmonellosis, and papilloma virus infection (Santos et al., 2001, Borzacchiello and Roperto, 2008, Van Rhijn et al., 2008). Therefore, cattle would be the natural animal model to investigate pathogenicity and disease dynamics, therapy and vaccine development of these diseases at the population level, as factors that contribute to animal disease outbreaks are similar to human settings such as close contact, crowding, contaminated fomites, and poor hygiene, commonly observed in cattle farms and facilities with a high number of people in relative small spaces, for instance in hospitals, schools, day care facilities and dense urban areas (Larson, 2007, Lanzas et al., 2010). Outcomes from these research may prove to be more relevant compared to the use of surrogate host models like rodents, which are infected in laboratory settings (Lanzas et al., 2010). A good example is the Bacillus Calmette Guérin (BCG) vaccine against human tuberculosis, developed in 1921 based on *Mycobacterium bovis*, the bacterium that causes bovine tuberculosis, as the pathogenesis of tuberculosis in human and cattle are very similar (Oettinger et al., 1999). Cows were also used in comparative studies in reproduction, lactation, and growth to elucidate related physiological processes important to human health (Ireland et al., 2008). The success of

producing over 80% developmentally competent bovine oocyte-cumulus complexes from small antral follicles through *in-vitro* maturation (IVM), arose from research seeking to translate this technology to improve *in-vitro* fertilization (IVF) in women (Blondin et al., 2002). Mammalian models, including laboratory and farm animals, assumed that the degree of evolutionary relatedness reflects the degree of mechanistic similarities are relatively closer to human in evolutionary terms, compared to fruit fly (*Drosophila melanogaster*) and the nematode *Caenorhabditis elegans*, two organisms commonly used in biological research. Therefore inference of knowledge between human and mammals has a higher probability to be relevant than with insects and nematodes (Carlson, 2012).

In the current study, a comparison of adipose tissue (AT) transcriptomic expression of cattle, pigs and elephants was performed, identifying differentially expressed genes (DEG) between breeds in cattle and pigs, dietary protein levels in cattle, also between sexes and ages in African elephants. The main objective of this study was to explore the potential role of AT in the differences of reproductive performance between different breeds of cattle (dairy versus beef) and pigs (Western versus Asian) with dissimilar levels of adiposity as AT has been reported to influence fertility in human and animals (Frisch, 1984, Day et al., 1986, Armstrong and Britt, 1987, Frisch, 1987, Tatman et al., 1990, Wade and Schneider, 1992, Wade and Jones, 2004). Related to this, dairy cows have experienced declining fertility in the last 50 years (Lucy, 2001, Dillon et al., 2006). Similarly, the captive populations of African elephants have long faced fertility issues and reports have suggested that obesity may be one of the causes of this problem (Clubb and

Mason, 2002, Brown et al., 2004, UAB, 2014). Despite advances made over the years in terms of oestrous cycle monitoring through hormone profiles, assessment of reproductive using ultrasonography, and development of assisted reproductive techniques such as artificial insemination, the reproductive performance of captive African elephants have not improved tremendously (Wasser et al., 1996, Brown et al., 1999, Hildebrandt et al., 1999, Hermes et al., 2000, Hildebrandt et al., 2006a, Thongtip et al., 2009). The Large White (LW) pigs are the most popular pig breeds in the UK pig industry, although they are not facing fertility issues, comparatively they are less prolific compared to the Meishan (MS) pigs, an Asian breed (Bolet et al., 1986, Bazer et al., 1988b, Hunter et al., 1993, Lee et al., 1995, Haley et al., 1995). The MS pigs are unlikely to be an important breed in the pig industry due to their slow growth and high adiposity (Borg et al., 1993, Johnson et al., 1995). However, the interest of comparing AT transcriptomic expressions between MS and LW breeds in the current study was to investigate possible avenues to further improve productivity of the LW pigs. In many instances throughout this dissertation, outcomes from transcriptomic research in human, rodent models and other animal species were related to results obtained in the current study. This approach is feasible based on the concept of homology, which proposes that the functions of genes in two or more species are presumed to be comparable if the sequence and structure of the genes share a common evolutionary origin (Doolittle, 1981, Abouheif et al., 1997, Pearson and Sierk, 2005, Emes, 2008). It is often assumed that conservation of gene function across species is more probable than functional divergence in related genes and convergence in unrelated genes (Abouheif et al., 1997, Wray and

Abouheif, 1998, Fitch, 2000). However, this may not always be true for all genes across different species. For this reason, it is necessary to be cautious when interpreting results.

Next generation sequencing (NGS), and more specifically for this study, RNA sequencing (RNA-seq), was used to examine differential transcriptomic expression of AT between animals as it provides an unbiased assessment of all transcripts with a greater dynamic range, and quantification of expression levels would not be limited to a range of signal intensities as compared with microarray approaches (Wang et al., 2009). Furthermore, the gene composition on currently available commercial bovine and porcine microarray chips are based on reference genome released in 2009 (Agilent, 2015a, Agilent, 2015b, Affymetrix, 2015), which have been superseded by current genome sequences. Furthermore commercial microarray assays for wildlife species such as the African elephant are not available. With RNA-seq, the latest genome assemblies on almost all mammal species are freely accessible and can be downloaded and used as reference genomes, in this case the cattle, pig and African elephant assemblies were updated between 2011 and 2012 (Ensembl, 2015a, Ensembl, 2015b, Ensembl, 2015c).

1.2 Adipose tissue

The AT is a highly specialised connective tissue found in discrete depots, and is made up of two predominant forms: brown adipose tissue (BAT) and white adipose tissue (WAT); (Gesta et al., 2007, Cannon and Nedergaard, 2008). The BAT has thermogenic properties through actions of uncoupling protein 1

(*UCP1*) in modulation of energy dissipation by release of heat through uncoupling mitochondrial respiration (Cannon and Nedergaard, 2004). This mechanism protects animals from hypothermia via classical non-shivering thermogenesis, as well as cold acclimation-recruited norepinephrine-induced thermogenesis (Cannon and Nedergaard, 2004). The WAT functions in maintenance of energy homeostasis of the body through controlled storage of triglycerides and their release in the form of fatty acids (FA) and glycerol (Smith, 1985). In addition, WAT provides insulation, mechanical protection and maintenance of body contour and structure in the form of connective tissue (Bauer-Kreisel et al., 2010). A subpopulation of WAT were discovered to exhibit characteristics and gene expression similar to that of BAT, and these cells became more prominent when subjected to prolonged cold stimulation, or when treated with β3-adrenergic receptor agonists that elevated intracellular cyclic adenosine monophosphate (c-AMP) that in turn increased mobilisation of FA reserves (Sutherland and Robison, 1969, Young et al., 1984, Loncar et al., 1988a, Loncar et al., 1988b, Cousin et al., 1992, Wu et al., 2012). These cells are termed beige adipocytes, and their thermogenic properties have been shown to carry much potential for establishing therapeutic possibilities to overcome metabolic diseases, including obesity (Harms and Seale, 2013). As WAT is the focus of the current study, the following discussion on AT will specifically refers to WAT.

1.2.1 Anatomy and physiology of white adipose tissue

The WAT appears white in colour as adipocytes contain a large unilobular lipid droplet that can fill up to 90% of the cell cytoplasm, the nucleus is displaced to the peripheral border of the cell and organelles such as the mitochondria are elongated (Henrikson et al., 1999). At a cellular level, mature white adipocytes represent from 33 - 66% of the total number of cells in the WAT. Other important cellular components of the AT are the endothelial cells, pericytes, smooth muscle cells, fibroblasts, committed adipose progenitor cells (preadipocytes), mesenchymal stem cells (MSCs), nerves, and macrophages. These cells are collectively termed as the stromal vascular fraction of the tissue (Gesta et al., 2007, Bauer-Kreisel et al., 2010).

Fat stored within white adipocytes is composed primarily of triglycerides, which comprises of three FAs attached to a backbone of a single glycerol with ester linkages (Nelson and Cox, 2004b). Glycerol is a simple sugar alcohol compound found in oils from plant and animal tissue, while FA is a chain of carbon atoms bonded with hydrogen atoms, and a terminal carboxylic acid group (Nelson and Cox, 2004a, Nelson and Cox, 2004b). Depending on their composition, FAs can be classified into two major groups: saturated and unsaturated. Saturated fatty acids (SFAs) do not contain any double bonds along the hydrocarbon chain, while FAs with double bonds are termed unsaturated (Roche, 1999). Unsaturated FAs can be further divided into monounsaturated fatty acids (MUFAs) with one double bond, and polyunsaturated fatty acids (PUFAs) with two or more double bonds (Roche, 1999). Positions of the hydrogen atoms at each end of the double bond give

rise to two distinctive geometric configurations termed *cis* and *trans* (Roche, 1999). Hydrogen atoms are on the same side of the hydrocarbon chain in the *cis* configuration, whereas they are oriented in the opposite direction of the chain in isomers with *trans* double bonds (Roche, 1999). PUFAs can be subdivided into two categories: the n-3 and the n-6 series (more commonly known as ω-3 and ω-6). This classification is determined by the location of the first double bond, numbered from the non-carboxyl end (Simopoulos, 1991). The n-3 and n-6 PUFAs are termed essential FAs as these cannot be synthesised by higher organisms. For this reason essential FAs must be obtained through food in these species (Simopoulos, 1991).

The most efficient storage of excess energy intake is through fat deposition, and the most direct route for lipid filling by adipocytes is through uptake of FAs from dietary sources (Rosen and Spiegelman, 2006). Food digestion in the intestine releases triglycerides from ingesta, and these triglycerides are then hydrolysed into FAs and monoglycerides. After crossing the intestinal epithelial cells into the intestinal lymph and the blood vessels, these FAs and monoglycerides are reincorporated into triglycerides, and are transported to the liver and AT in the form of lipid droplets known as chylomicrons (Guyton and Hall, 2006, Dixon, 2010). Both adipose and liver tissue contain large quantities of the enzyme lipoprotein lipase (LPL) that hydrolyses triglycerides from chylomicrons and release FAs and glycerol. The FAs that diffuse into adipose and liver tissue are incorporated into triglycerides by condensing with glycerol within the adipocytes and hepatocytes (Guyton and Hall, 2006, Cunningham and Klein, 2007). In times of excess carbohydrate intake, triglycerides can be also be synthesised from carbohydrates and stored as fat

(Guyton and Hall, 2006). Carbohydrates are converted into acetyl-CoA through glycolysis during glucose metabolism. Acetyl-CoA then undergoes a two-step process with malonyl-CoA, a product from carboxylation of acetyl-CoA by the enzyme acetyl-CoA carboxylase (ACACA), and nicotinamide adenine dinucleotide phosphate (NADPH) to form FA (Guyton and Hall, 2006). Once the synthesised FAs have elongated to contain 14 to 18 carbon atoms, they can then bind with glycerol derived from α-glycerophosphate that is produced during glucose degradation via glycolysis, to form triglycerides (Guyton and Hall, 2006). Stored fat in adipocytes must be hydrolysed into FAs and glycerol before it can be transported out of AT to other tissues in the form of free fatty acids (FFAs), also known as non-esterified fatty acids (NEFAs). On leaving adipocytes, FAs combine with albumin in the plasma, thus forming FFAs (Guyton and Hall, 2006).

1.2.2 White adipose tissue depots

WAT is found in several depots throughout the body, and these depots can be classified into two major categories, visceral and peripheral, located in the intra-abdominal and subcutaneous regions respectively (Gesta et al., 2007). The intra-abdominal fat depots are predominantly situated surrounding the omentum, intestines and peri-renal area. By contrast, the subcutaneous fat depots are found in the posterior area of the pelvic region, lower limbs (especially for primates) and around the abdomen (Cinti, 2001, Hausman et al., 2001). However, the different WAT depots have distinct morphologies, metabolic properties and functions (Rosen and MacDougald, 2006, Gesta et

al., 2007). This may be due partly to a divergence in transcription factors and gene expression patterns (Vidal, 2001, Vohl et al., 2004, Gesta et al., 2006, Tchkonia et al., 2007, Tchkonia et al., 2013).

Human visceral adipocytes have been shown to contain higher levels of glucocorticoid receptor (GR) expression at the protein and mRNA level as compared to subcutaneous adipocytes (Rebuffe-Scrive et al., 1990). Given that GR activation in fat cells can cause an increase in *LPL* expression, which enhanced triglyceride storage (McCarty, 2001), it is possible that visceral adipocytes are more inclined to lipid deposition than subcutaneous adipocytes due to higher *LPL* activity. Conversely, omental adipocytes were reported to have twice the level of β-adrenergic receptor expression (Rebuffe-Scrive et al., 1989, Arner et al., 1990, Hellmer et al., 1992), but lower α2-adrenergic receptors compared to subcutaneous adipocytes in primary cell cultures (Vikman et al., 1996). Because β-adrenergic receptors stimulate lipolysis while the active α-adrenergic receptors inhibit lipolysis, this suggests that omental adipocytes are more "primed" for lipolysis compared to subcutaneous adipocytes (Collins and Surwit, 2001, Garenc et al., 2002). Furthermore, omental adipocytes express 5 – 10 times greater sensitivity towards the lipolytic effects of catecholamine (Hellmer et al., 1992). In addition, although the number of insulin receptors is significantly higher in omental AT than subcutaneous AT, omental AT are less responsive to the anti-lipolytic effect of insulin compared to subcutaneous AT (Bolinder et al., 1983). This is likely due to less efficient insulin signalling of the insulin receptor found in omental AT (Lefebvre et al., 1998).

Mesenteric fat cells receive greater volumes of blood supply than subcutaneous cells (Crandall et al., 1984). Consistent with this, omental AT contains a high proportion of endothelial cells in the stromal-vascular cell fraction whereas subcutaneous AT appears almost devoid of endothelial cells (Hauner et al., 1989). This suggests that abdominal adipose depots may receive higher levels of humoral factors related to regulation of AT development, more substrate for lipid accumulation and easier access to the circulation at times of FA mobilisation. These results indicated that adipocytes from the visceral region are more metabolically active, with higher lipid storage and mobilisation capabilities than those in the subcutaneous region. It is possible that visceral adipose depots respond in a more timely fashion to changes in systemic energy balance, and be able to better reflect metabolic status of the subject of research. Thus, in the current study AT from visceral depots were used, unless tissue from these sites were not sufficient for analysis such as in neonate pigs as described later.

1.2.3 Development of adipose tissue

Adipocytes are generally believed to have derived from MSCs which originate from the mesoderm (Sakurai et al., 2006). The MSCs have been described to possess self-renewal properties and are potentially capable of differentiating into any mesenchymal cell types such as osteoblasts, chondrocytes, haematopoietic cells, endothelial cells and smooth muscle cells, besides adipocytes (Billon et al., 2008). Differentiation of mouse embryonic stem cells (mESCs) towards the adipocyte lineage can be achieved by treating with all-

trans retinoic acid (ATRA) and subsequently adipogenic factors such as insulin, triiodothyronine, and rosiglitazone (Dani et al., 1997). However, through the use of neuroepithelial progenitors derived from genetically engineered mESCs and transgenic mice produced through Cre-mediated recombination, the neural crest was shown to give rise to adipocytes in the face and neck region (Billon et al., 2007). This suggests that under certain circumstances, distinct stem or progenitor cell populations may contribute to adipose lineages (Billon et al., 2007, Billon and Dani, 2012).

Dissimilar development of AT has been observed between humans and different animal species. In human embryos, the appearance of white adipocytes are indicated by the presence of fat lobules, the earliest structures seen prior to the development of the typical vacuolated fat cells (Poissonnet et al., 1983). The fat lobules are observed around the 14 - 16th week of gestation in the head region (Poissonnet et al., 1984). These lobules progressively appear in the trunk, followed by the limbs. By the age of 28 weeks, fat lobules can be detected in all presumptive visceral and subcutaneous WAT locations (Poissonnet et al., 1984). A similar progression of WAT differentiation is observed during fetal development in the pig (Hausman and Richardson, 1982, Hausman, 1985). The BAT was described to be the prominent fat tissue in bovine foetuses (Alexander et al., 1975, Landis et al., 2002), however later studies revealed that in the last trimester of gestation, more abundant fat cells resembling the unilocular white adipocytes were observed (Taga et al., 2012b). By contrast, WAT in rodents develops mainly after birth and cannot be macroscopically detected during embryogenesis. The first appearance of WAT was observed to be in the

perigonadal and subcutaneous depots, followed by the omental depot (Billon and Dani, 2012).

The enormous capacity of AT to expand in volume and cell numbers is an important characteristic in maintaining energy balance in the body system through lipogenesis and lipolysis of the lipids in adipocytes postnatally (Spalding et al., 2008). Previously it was thought that an increase in the number of adipocytes occurred only in the early period of life in humans, within the first few years of life, and then again from age 9 to 13 years (Salans et al., 1973). Also, it was believed that cell numbers established in childhood remained constant throughout adulthood (Knittle et al., 1979). However, subsequent studies have shown that quantitative increment of adipocytes continues into adulthood in humans and animals (Hirsch and Batchelor, 1976, Faust et al., 1978, Bertrand et al., 1980, Bertrand et al., 1984, Spalding et al., 2008, Drolet et al., 2008).

Expansion of AT due to an increment of the size of adipocytes is referred to as hypertrophy, typically due to increased deposition of lipid into the cytoplasm of existing adipocytes (Salans et al., 1973, Jo et al., 2009). Enlargement of fat mass can also be due to an elevation of adipocyte numbers (hyperplasia) from proliferation and differentiation of preadipocytes (Salans et al., 1973, Jo et al., 2009). Lipid filling of adipocytes is accomplished by direct uptake of FAs from dietary fats or via *de novo* lipogenesis, where FAs are synthesised from glucose through glycolysis and tricarboxylic acid (TCA) cycle, and lipids are then stored in lipid droplets within the adipocytes (Kersten, 2001). On the other hand, adipogenesis is a two-step process that involved the transition of non-

committed progenitors into mature adipocytes, thus increasing the number of adipocytes (Farmer, 2006, Rosen and MacDougald, 2006, Billon et al., 2008).

The first step of adipogenesis is known as the determination phase where adipocyte precursors, or preadipocytes, are generated from MSCs (Farmer, 2006, Rosen and MacDougald, 2006, Billon et al., 2008). These cells are committed towards the adipocyte lineage and believed to be unable to differentiate into other mesenchymal cell types. The second step involves the terminal differentiation of preadipocytes into mature functional adipocytes (Farmer, 2006, Rosen and MacDougald, 2006, Billon et al., 2008). Results from studies in human and rodent obesity models indicate that adipocyte hypertrophy often precedes adipocyte hyperplasia (Faust et al., 1978, Maumus et al., 2008). In fact, enlarged adipocytes appear to secrete preadipocytes differentiation factors that could promote adipogenesis in cell cultures (Considine et al., 1996, Lai et al., 2012).

Different mechanisms are involved in hypertrophic and hyperplastic expansion of AT. In brief, lipogenesis commences with the acquisition of exogenous FA by extracellular lipolysis of triglyceride from chylomicrons and very low density lipoproteins (VLDL) through the enzymatic activities of LPL (Goldberg et al., 2009, Zaidi et al., 2013). Alternatively, glucose from digestion and hydrolysis of dietary carbohydrates, is used as the main source of carbon molecules for *de novo* lipogenesis (Kersten, 2001). Pyruvate is produced from glucose through glycolysis and enters mitochondria, it is then converted into acetyl-CoA via the TCA cycle and exported to cytosol. Acetyl-CoA in the cytosol is used as precursor for FA synthesis catalysed by enzymes such as acetyl-CoA

carboxylase α (ACACA), fatty acid synthase (FASN), and stearoyl-CoA desaturase (SCD); (Kim and Spiegelman, 1996, Kersten, 2001, Strable and Ntambi, 2010, Li and Cheng, 2014). However, dietary fats are the preferred substrate for synthesis of new structural lipids over carbohydrates as the latter is a more energy consuming process (Hellerstein, 2001, Menendez and Lupu, 2007). Finally, FAs produced from these processes enter adipocytes via fatty acid translocase (CD36), the FA uptake channel and then form lipid droplets (Goldberg et al., 2009).

Differentiation of committed precursor cells appears to be mediated through a pathway encoded by transcription factors such as CCAAT/enhancer binding proteins (*C/EBP*) β, *C/EBPδ*, *C/EBPα*, peroxisome proliferator activated receptor γ (*PPARγ*), and sterol regulatory element binding transcription factor 1 (*SREBF1*); (Freytag et al., 1994, Tontonoz et al., 1994, Hu et al., 1995, Yeh et al., 1995, Rosen et al., 1999, Rosen et al., 2000, Rosen et al., 2002). The regulatory relationship between *C/EBP* family members and *PPARγ* is complex (Tontonoz et al., 1994, Rosen et al., 1999, Rosen et al., 2000). *C/EBPβ* and *C/EBPδ* are necessary to induce expression of *PPARγ* in a linear cascade of adipocyte differentiation, including activation of *C/EBPα* (Tontonoz et al., 1994, Rosen et al., 1999, Rosen et al., 2000). A positive feedback loop exists between *C/EBPα* and *PPARγ*, thus *PPARγ* triggers *C/EBPα*, and in turn *C/EBPα* is necessary to activate expression of *PPARγ* in differentiated adipocytes (Wu et al., 1999). Rosen and colleagues (2002) observed that *C/EBPα* fails to induce any lipid accumulation in *PPARγ*$^{-/-}$ adipocytes differentiated from fibroblasts in mice, yet ectopic expression of *PPARγ* can stimulate adipogenesis in *PPARγ*$^{-/-}$ fibroblasts. Therefore, it was proposed

that the role of *C/EBPα* in fat cell differentiation is limited to induction and maintenance of *PPARγ* levels, and that *C/EBPα* alone could not support the adipogenic transition of preadipocytes (Rosen et al., 2002). In addition, *C/EBPα* was also reported to be important for the preservation of insulin sensitivity in mature adipocytes, as fat cells lacking *C/EBPα* exhibited a reduction of insulin-sensitive glucose transport (Wu et al., 1999). *PPARγ* is especially important in the final stage of conversion of preadipocytes into differentiated adipocytes (Fernyhough et al., 2007). Similarly *SREBP1c* stimulates differentiation of cultured preadipocyte cell lines, and actively promotes expression of many of the genes necessary for lipogenesis *in vivo*, including *LPL*, *ACACA*, *FASN*, and *SCD* (Kim and Spiegelman, 1996, Strable and Ntambi, 2010). The indispensable role of *PPARγ* in adipogenesis was also demonstrated *in vivo* in chimeric mice with *PPARγ* null cells showed little or zero lipid accumulation in fetal tissues, and *PPARγ*-deficient pups exhibited a lack of AT (Barak et al., 1999).

Heterodimers are formed between *PPARγ* and retinoic X receptors (*RXR*) in order to initiate adipogenic activities of AT in murine cell line (Graves et al., 1992, Choong et al., 2015). This was further demonstrated by genome-wide binding profile of *PPARγ* and *RXR* using chromatin immunoprecipitation combined with microarray assays or DNA sequencing where high degree of overlap between *PPARγ* and *RXR* binding on DNA was reported (Nielsen et al., 2008, Lefterova et al., 2008, Hamza et al., 2009). Adipogenic activities are inhibited *in vivo* in *PPARγ* and *RXRα* deficient murine foetuses with decreased lipid droplets abundance and size in comparison with wild type foetuses (Barak et al., 1999). Furthermore, PPARγ/RXR heterodimers were also discovered to

affect adiposity of mature adipocytes as Yamauchi and colleagues (2001) found that *RXR* and *PPARγ* antagonists decrease triglyceride content in WAT thus reducing high-fat diet induced obesity in mice.

1.3 Reproduction

The importance of reproductive efficiency cannot be overstated. Not only is efficient reproduction required for species sustainability, improvements in reproductive efficiency are important for enhancing the sustainable intensification of food animal production. For this reason, the current study examines AT function at the molecular level and infers mechanistic links to the reproductive characteristics of the species examined. Therefore it is necessary to critically consider the evidence supporting mechanistic links between the regulation of reproductive function and AT metabolism.

1.3.1 Regulation of reproductive functions by the hypothalamic-pituitary-gonadal axis

The principal hormones regulating reproduction are produced by hypothalamus, anterior pituitary gland, and the ovaries and testicles (collectively known as gonads), forming the hypothalamic-pituitary-gonadal (HPG) axis (Bulun and Adashi, 2003). Neurosecretory cells of the hypothalamus synthesise and release gonadotropin-releasing hormone (GnRH) in a pulsatile fashion into the hypothalamic-hypophyseal-portal circulation (Millar et al., 2004). GnRH in turn stimulates gonadotropes in the

anterior pituitary to synthesise and release the gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH), both of which control gonadal functions. In the females, ovarian follicles are stimulated by FSH to develop and mature, while LH stimulates ovulation and *corpus luteum* (CL) formation (Klein, 2003, Charlton, 2008, Meethal et al., 2009). As the focus of the current study was on effect of changes in AT on reproductive functions in female animals, the following section will centre on hormonal regulation of female reproductive functions.

The primary physiological source of estrogen is the granulosa cells of the ovarian follicles following FSH stimulation and the CL (Nelson and Bulun, 2001). In addition, estrogen is also produced, albeit in lower concentrations than the ovary, by peripheral issues such as AT, including that of the mammary gland. However, synthesis of estrogen in these tissues is stimulated by glucocorticoids, together with members of the interleukin-6 cytokine family (Simpson, 2003). On the other hand, the CLs in the ovaries are the major site of progesterone (P_4) production, formed after the release of a matured oocyte from an ovarian follicle during ovulation following a LH surge (Bulun and Adashi, 2003). P_4 is also produced from ovarian cumulus cells as a result of hormonal stimulation from both LH and FSH (Shimada and Terada, 2002, Chian et al., 1999).

The hypothalamus, pituitary gland and the gonads form a complex feedback loop to regulate the secretion of reproductive hormones. During the follicular phase of oogenesis, the relatively low circulating concentrations of estrogen has a negative feedback effect on hypothalamic GnRH and pituitary

gonadotropin secretion (Bulun and Adashi, 2003). Estrogen continues to rise, and when it has reached a threshold level, this triggers positive feedback on the hypothalamic GnRH and pituitary gonadotropin secretions, inducing a LH surge resulting in ovulation of a secondary follicle, and as a consequence a CL is formed (Bulun and Adashi, 2003). As P_4 levels begin to rise and proceed negative feedback to the hypothalamus and the anterior pituitary, this inhibits the estrogen-LH positive feedback loop. In cases of conception, the CL will continue to produce P_4 until the placenta has developed sufficiently to synthesise P_4 and the high level of P_4 prevents further follicular development and ovulation (Bulun and Adashi, 2003). If conception does not occur, CL will regress and the concomitant decreased secretion of P_4 will allow the hypothalamus to resume secretion of GnRH and stimulates production of gonadotropin by the anterior pituitary (Bulun and Adashi, 2003).

1.3.2 Links between reproductive functions and adipose tissue

Reproduction is one of the most energy expensive activities that occur in female mammals, involving ovulation, conception, maintenance of one or more foetuses throughout pregnancy, and lactation to provide nutrients to the neonates (Wade and Schneider, 1992, Wade et al., 1996). As a general rule, reproductive activities are more successful when energy is plentiful than when it is scarce (Wade and Schneider, 1992). In fact, an increase in food intake and accumulation of AT have been recorded in many species during gestation in preparation for the birth and milk production for sustenance of the offspring (Richard and Trayhurn, 1985, Wade and Schneider, 1992). Conversely,

obesity has been linked with infertility due to deficits in the availability of fuels for intracellular oxidation as a result of insulin resistance, even when there is an apparent excess storage of energy in AT (Wade and Jones, 2004). This suggests that there is an optimal level of adiposity required for optimal reproductive activity to occur.

Suppression of puberty and fertility due to low body fat composition has been demonstrated in both human and animals. Frisch and colleagues (1974, 1984, 1987) identified that a threshold ratio of fat to lean mass is necessary for puberty, and for maintenance of female reproductive ability in humans. They also found that weight loss in the range of 10 - 15 % of normal weight for height can delay menarche and cause amenorrhoea. Hamsters with high body fat can resist fasting induced anoestrus compared to animals with low body fat (Schneider and Wade, 1990). Dietary restriction (60% of maintenance energy) over a period of eight months resulting in a low body condition score (BCS) in ewes caused a reduction in LH and GnRH synthesis and release compared to ewes on 100% maintenance diet (Tatman et al., 1990). Similarly, sexually matured gilts fed with a caloric and protein restricted diet (8.5% calories and 33% protein intake of control group) experienced anoestrus 46 $\pm$ 9 days after feed restriction was imposed, in correlation with a significant loss of body weight and back fat thickness. Gilts only returned to oestrus, with an increase in concentration and frequency of LH release, as a consequence of significant weight gain following increased feed intake (Armstrong and Britt, 1987). Heifers maintained on a low-energy diet (reduced energy level to produce 200g body weight gain/day compared to 900g body weight gain/day for the control diet) failed to attain puberty and were unable to exhibit the required

increase in LH pulse frequency seen in heifers on regular diet starting from the first oestrus exhibited by heifers on the control diet (Day et al., 1986). Thus, AT appeared to serve as a buffer against nutritionally induced infertility, until the depot has been depleted when nutritional deprivation continues (Wade and Schneider, 1992).

1.3.3 Adipokines in reproduction

Hormones secreted by AT are termed adipokines to reflect their initial discovery in AT, although it was later recognised that adipokines are also secreted by other tissues (Kershaw and Flier, 2004). Adipokines are specialised cytokines which elicit specific effects on target tissues (Trayhurn et al., 1999, Tsao et al., 2003). A number of adipokines have been discovered, including adiponectin, resistin, leptin (LEP) and tumour necrosis factor-α (TNF-α); (Ahima and Flier, 2000, Moller, 2000, Steppan et al., 2001, Trayhurn and Beattie, 2001). It has been suggested that adipokine expression in AT are primarily under the regulation of *PPARγ*, an important transcription factor involved in adipocyte differentiation as described above (Hofmann et al., 1994, Hollenberg et al., 1997, Iwaki et al., 2003, Tomaru et al., 2009). Among the adipokines listed above, there has been extensive research on functional reproductive mechanisms for *LEP* in relation to AT and energy, and it was a major gene of interest throughout the current study.

1.3.3.1 Leptin

The adipokine, LEP is primarily secreted from white adipocytes, and serum levels of LEP have been correlated with the size of AT mass in human and animals (Shimizu et al., 1997a, Barb et al., 2001, Williams et al., 2002, Ruhl et al., 2007, Shan et al., 2008, Harris, 2013). It was first described to bind to a specific leptin receptor (*LEPR*) found in the satiety centre of the hypothalamus and LEP causes a reduction of food intake and augmentation of energy expenditure, thus preventing overload of lipid accumulation in adipocytes (Coleman, 1973, Coleman, 1978). Another mechanism whereby LEP can effect a decline in triglyceride deposition is through inhibition of insulin synthesis in pancreatic-β cells (Seufert, 2004). This is likely to be important in reproductive activities, as both obese and malnourished mammals face infertility issues (Burks et al., 2000). Obesity has been related to hyperinsulinemia which can contribute to premature arrest of follicle growth, thus leading to anovulation, one of the clinical signs of polycystic ovary syndrome (PCOS) in women (Franks et al., 1999). On the other hand, insulin was observed to influence concentration of plasma LEP in lactating cows (Block et al., 2001, Block et al., 2003). Research showed that hyperinsulinemia in lactating Holstein cows induced an increase in plasma LEP concentration, and reduction of plasma insulin in undernourished lactating dairy cows resulted in lower plasma LEP (Block et al., 2001, Block et al., 2003). This may partly explain the reasons behind difficulties of high producing dairy cows in negative energy balance (NEB) to resume normal oestrous cycles and the low insemination success rate (Wathes et al., 2012).

LEP was discovered to influence the reproductive axis by signalling between AT and the neuroendocrine axis, since proteins associated with appetite found in the hypothalamus, such as neuropeptide Y, pro-opiomelanocortin (POMC) and kisspeptin, mediate the functions of LEP, and *vice versa* through interneuron pathways (Chaldakov et al., 2010, Hausman et al., 2012). Because LEP levels are influenced by the fat pad mass, which is an indirect indication of the availability of nutrients, fasting was shown to cause a decrease in LEP and LH levels, and LH pulsatility was restored after exogenous LEP administration (Nagatani et al., 1998).

It was hypothesised that an elevation in LEP to a threshold level brought about the activation of the HPG axis and the onset of puberty. In humans, this was found to be true in girls, but not in boys (Horlick et al., 2000). In beef heifers, marked increases in both circulating LEP and *LEP* gene expression occur during pubertal development and are associated with increases in serum insulin-like growth factor 1 (IGF-I) and body weight (Garcia et al., 2002). Conversely, chronic administration of LEP in beef heifers failed to induce puberty or alter endocrine characteristics nearing the time of expected puberty (Maciel et al., 2004). Maciel et al. (2004) suggested that these discrepancies may be explained by increased LEPR in the ventromedial hypothalamus cause by episodes of malnutrition (Dyer et al., 1997), and reports have suggested that LEP may only depress LH secretion during nutritional stress in cattle (Amstalden et al., 2002). Therefore, exogenous LEP did not affect LH secretion patterns in well-fed heifers (Amstalden et al., 2002). In pigs, LEP levels, hypothalamic *LEPR* mRNA and estrogen-induced *LEP* mRNA expression in fat increased with age and adiposity, and this occurred at the

time of expected puberty (Barb et al., 2005). Serum and milk LEP concentrations in lactating sows were revealed to correlate positively with backfat thickness and level of dietary energy fed during gestation as well as feed consumption (Barb et al., 2005).

LEP signals are detected by the central nervous system and transmitted by the neuroendocrine system into signals which ultimately regulate GnRH release from the hypothalamus, and LH and FSH from the pituitary gland, and thereby influence ovarian follicular development and steroidogenesis (Hausman and Barb, 2010). However *LEP* and *LEPR* expression have been detected in the reproductive tissues, suggesting potential direct LEP-related mechanisms in pregnancy, implantation and embryo development (Budak et al., 2006, Cervero et al., 2006). Furthermore *LEPR* mRNA was also detected in bovine AT (Ren et al., 2002, Chelikani et al., 2003), and localised lipolytic action of LEP on adipocytes was demonstrated by depletion of denervated fat pads in hyperleptinemic rats (Wang et al., 1999b).

1.4 Hypothesis and significance of study

The current study aimed to establish transcriptomic signatures of AT in three different species of animals, cattle, pigs and African elephants. The dairy and pig industries are important in the United Kingdom (UK), not only in supplying milk and meat for local consumption, they also contribute significantly to UK economy through export of dairy products and pork (DEFRA, 2014, DEFRA, 2015b). However, reproductive performance of dairy cattle is reportedly in long term decline till date (Royal et al., 2000, Lucy, 2001, Bousquet et al., 2004,

Dillon et al., 2006, Macdonald et al., 2008). Indeed dairy cow fertility had been declining over the decades in UK and elsewhere. One possibility is that high milk production may have led to the decline in fertility of dairy cows (Royal et al., 2000, Lucy, 2001, Bousquet et al., 2004, Dillon et al., 2006, Macdonald et al., 2008, Bowmana et al., 2012). One of the major fertility challenges faced by the cattle industries is sub-optimal conception rates. In 2007, only 8.5% of beef heifers, and 6.9% dairy cattle had calved by 24 months, the target age in both industries (CHAWG, 2014). The average calving interval was 403 days for beef herds, and 428 days for dairy herds (CHAWG, 2014). As the aim is to produce one calf per cow per year, any deviation from this decreases profit and reduces efficiency. However, the most distinctive fertility disparity is the number of live births between dairy and beef cows. The annual birth rate of calves from dairy cows in 2011 and 2012 was between 38.32 and 40.33%, while the birth rate was more than double from beef cows during the same period, between 88.87 and 88.38% (CHAWG, 2014, DEFRA, 2015b). Similar challenges are seen in the pig industry where the predominant European Large White (LW) commercial pig breeds exhibit impaired fertility as compared to the Asian counterparts. For example, the Asian Meishan (MS) pig breed has high adiposity and has been reported to perform better reproductively compared to the contemporary western pig breed, LW (Bolet et al., 1986, Bazer et al., 1988a, Bazer et al., 1988b, Bidanel et al., 1990, White and Wheeler, 1995, Haley et al., 1995). The MS pigs were reported to attain sexual maturity 105 days earlier, have higher ovulation rate, higher number of viable embryo during early pregnancy, lower conceptus mortality rate, and higher number of live births compared to the LW (Bolet et al., 1986, Bazer et al.,

1988b, Hunter et al., 1993, Lee et al., 1995, Haley et al., 1995). While elephants may not be commercially important as the food animals, they are iconic animals in the wild and in captive facilities, and play a major role in public education on wildlife and habitat conservation, as well as in ecotourism (Saunders and Young, 1985, Iversen, 1996, Smith and Hutchins, 2000). Unfortunately, infertility has long been an issue faced by captive African elephants, and one of the reasons suggested was that obesity in these animals could lead to irregular reproductive cycles and reproductive tract pathologies (Clubb and Mason, 2002, Brown et al., 2004). In recent studies, body condition score (BCS) of non-cycling female zoo African elephants were generally higher than the ones with regular oestrous cycles (Morfeld and Brown, 2014, Morfeld et al., 2014). Furthermore, non-cycling animals with obese BCS were shown to have elevated serum leptin and insulin levels compared to that of their cycling counterparts (Morfeld and Brown, 2014, Morfeld et al., 2014), commonly observed in obese and anovulatory women with polycystic ovarian syndrome (Chakrabarti, 2013), thus suggesting that ovarian acyclicity in zoo African elephants is associated with body condition indicative of obesity (Morfeld and Brown, 2014, Morfeld et al., 2014). Conversely, animals in the wild population were not reported to suffer from such complications, although they are facing threats from poachers and habitat destruction (UNEP et al., 2013, WWF, 2013, Wittemyer et al., 2014). Therefore, for all of these reasons, one of the objectives of this study was to examine the gene transcriptions of AT in these animals in hope to identify possible mechanism by which AT could influence fertility.

Important genes involved in adipogenesis and maintenance of adiposity in AT such as *PPARγ* and *C/EBPβ* are also expressed in the female reproductive tracts and potentially modulate reproduction functions (Bagchi et al., 2006, Froment et al., 2006, Mantena et al., 2006, Minge et al., 2008). The *PPARγ* expression is predominantly in the AT (Chawla et al., 1994, Chawla et al., 2001), however studies showed that mice with *Pparγ* specific deletions at ovaries and mammary glands, epithelium (including uterine epithelium), and in other secretory tissues using Cre-loxP recombination system resulted in reduced fertility due to decreased P_4 secretion from the ovaries and a lower number of embryo implantation (Cui et al., 2002). Furthermore, *PPARγ* protein and mRNA expressions have been detected in ovarian granulosa cells of rats and sheep, and were observed to promote follicular development, and preserved CL function during pregnancy (Komar et al., 2001, Froment et al., 2003, Froment et al., 2006). Treatment of women with PCOS using PPAR-activating ligands such as thiazolidinediones (TZDs) showed increased ovulation and pregnancy rates (Froment and Touraine, 2006). This may be due to improved ovarian functions, but also as a result of anti-inflammatory action of *PPARγ* on AT of PCOS patients with chronic inflammation, as the number of macrophages were significantly reduced in mice ovaries treated with TZDs (Minge et al., 2006). On the other hand, *C/EBPβ* was found to play an important role in mediating proliferation and differentiation of the endometrium during decidualisation phase of embryo implantation through induction of estrogen and P_4 secretion (Bagchi et al., 2006, Mantena et al., 2006, Plante et al., 2009). *C/EBPβ* mRNA and protein levels were markedly increased at the time of endometrial receptivity in human (Plante et al., 2009),

and *C/EBPβ* null mice exhibited a complete lack of decidualisation when stimulated by mechanical perturbation of the endometrium and were infertile (Bagchi et al., 2006).

Close proximity of an appreciable amount of AT to the bovine female reproductive tract suggested paracrine activities of AT on the tract (MacDougal, 2012). This has also been proposed for humans (Mohamed-Ali et al., 1998), although mechanisms described were related to adipokines, adipocyte-derived proteins with endocrine functions, rather than transcription factors related to adipogenesis and lipogenesis (Ahima and Flier, 2000, Galic et al., 2010). Therefore, whether transcription of *PPARγ* and *C/EBPβ* and other factors in AT directly influence reproductive functions of human and animals remains to be elucidated.

As outlined above, mechanistic, physiological and genetic evidence suggests AT is a crucial regulator of reproductive efficiency in people and animals. This evidence supports the hypothesis of this study that AT transcriptome will have molecular signatures that influence reproductive function in animals. To test this hypothesis, RNA-seq was used to characterize the transcriptomes of AT of (i) dairy and beef cattle, (ii) Asian (MS) and European (LW) pigs, (iii) male and female African elephants and (iv) beef-dairy cross cattle fed with different levels of dietary protein, and were related to the known reproductive phenotypes of these species and breeds. As described earlier, both excessively low and high adiposity appear to be detrimental to fertility in both humans and animals (Frisch, 1984, Day et al., 1986, Armstrong and Britt, 1987, Frisch, 1987, Tatman et al., 1990, Wade and Schneider, 1992, Wade

and Jones, 2004). However, these reports were generally on humans or on laboratory rodent models, and research animals subjected to extreme NEB or metabolic imbalance conditions. In the current study, adipose samples were obtained from animals under regular management systems or from the wild, therefore the results obtained are likely to be more relevant to the conditions of these animals *in situ*.

Genome wide transcriptomic comparison between AT of different breeds in cattle and pigs in relation to adiposity, energy expenditure and reproductive performance has not been reported to date. The first transcriptome profile analysis of porcine AT by high-throughput deep sequencing was reported by Li and colleagues (2012a), and their results suggested differential adipogenesis capabilities between the Rongchang (obese) and Landrace (lean) pigs. A comparative transcriptomic study between AT of dairy and beef cattle breeds using NGS was published recently, but the outcome of the study was primarily descriptive, discussing the differences in gene expression, rather than functional application of results obtained (Thomson et al., 2013). Also, as such a study has yet to be carried out in the wild African elephant population, data from this study could serve as an invaluable source of information to be incorporated into management of the captive population. Results from this study, outlined in the following chapters, indicates potential roles for ATRA, vitamin D and protein in influencing the AT transcriptome. Therefore, successful translation of the knowledge found could potentially help to improve management of food animals and captive wildlife through modification of feed formulation. Moreover, a change in diet is more likely to produce faster results in the current population compared to selective breeding

or genetic manipulation. This is important in order to rapidly address global food security, where productivity could be increased through safe and sustainable means, such as dietary changes, thereby improving reproductive health in food animals. Also, breeding difficulties faced by captive wild animals need to be resolve quickly before the current population reach reproductive senescence and are no longer able to reproduce. Reports have suggested that given the current reproductive output of captive African elephant population, it is not self-sustaining, and African elephant will become extinct in captivity within the next 50 years if the situation does not improve (Olson and Wiese, 2000, Wiese and Willis, 2006, Brown, 2014).

Chapter 2 General Materials and Methods

2.1 Materials and methods

This chapter describes the entire workflow of RNA sequencing (RNA-seq) and analysis of data generated, from sample collection to pathway analysis (Figure 2.1) as well as validation of transcriptome expression by quantitative reverse transcription PCR (qRT-PCR). This workflow was common for all three species investigated in this study. Any additional procedures unique to the individual species were outlined in their respective chapters. Experimental protocols and operative procedures were reviewed and approved by the Research Directorate and the Ethics Committee at the School of Veterinary Medicine and Science, University of Nottingham.

2.2 RNA extraction and quality control

A total of 400mg adipose tissue (AT) was homogenised using gentleMACS M tubes (VWR International Ltd, Leicestershire, UK) with 1mL of QIAzol Lysis Reagent (Qiagen, Crawley West Sussex, UK) and a gentleMACS dissociator (VWR International Ltd, Leicestershire, UK). Homogenate was carefully pipetted into a new microcentrifuge tube and subsequent purification of total RNA was carried out using the Qiagen RNeasy® Tissue Mini Kit (Qiagen, Crawley West Sussex, UK). After the addition of chloroform, the samples were centrifuged (9,632g) for 15 minutes at 4°C to yield three phases (Heraeus PICO 17 Centrifuge, Thermo Scientific, Osterode, Germany). The upper, colourless, aqueous phase containing RNA was transferred to a new

microcentrifuge tube and added with approximately 600µL of 70% ethanol, and mixed thoroughly by vortexing (Evolution ZX FB15012 TopMix, Fisher Scientific, Leicestershire, UK). The sample was transferred to a RNeasy® Mini spin column and centrifuged (9,632g) at room temperature (15 – 25°C) for 15 seconds in a 2mL collection tube. Flow through was discarded. Buffer RW1 was added to wash the membrane, the spin column was centrifuged (9,632g) at room temperature for 15 seconds, and again flow through was discarded.

The sample was treated with DNase I at 25°C for 15 minutes to remove residual genomic DNA from the RNA. The spin column was then sequentially washed with Buffer RPE to ensure that ethanol was not carried over during RNA elution. This was followed by placing the spin column in a new 2mL collection tube and centrifuged (9,632g) at room temperature for 15 seconds to eliminate any carryover of Buffer RPE. The spin column was placed in another new 1.5mL collection tube, 33µL RNase-free water was added directly to the spin column membrane, and the spin column was centrifuged (9,632g) at room temperature for 1 minute to elute the RNA. This step was repeated with the eluate to increase the RNA yield.

RNA purity and concentration were determined utilising the NanoDrop ND-8000 Spectrophotometer (Labtech International Ltd., East Sussex, UK), by assessing the 260/280 nm absorption ratio > 1.8. This was followed by assessment of sample integrities using Agilent 2100 Bioanalyzer (Agilent Technologies UK Ltd., Stockport, UK). Samples with RNA integrity value (RIN number) lower than 7.0, and total RNA concentration below 100ng/µL were excluded from sequencing. This was to ensure that the quality and quantity of

total RNA was sufficient to generate cDNA libraries and subsequently transcriptome reads of good quality for downstream analyses.

2.3 RNA-sequencing

2.3.1 Preparation of cDNA libraries

Genome-wide RNA transcription was quantified using next generation sequencing (NGS) with the Illumina HiSeq 2500 platform, performed by Edinburgh Genomics, Roslin Institute, University of Edinburgh. The cDNA libraries were prepared with 1µg of total RNA with the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, USA). RNA was first purified to obtain poly-A containing mRNA, followed by fragmentation of mRNA for cDNA synthesis. The RNA template was removed during synthesis of second strand cDNA, and double stranded cDNA was produced. End repair process was carried out to eliminate overhangs produced at both 3’ and 5’ ends of cDNA due to fragmentation. The polymerase activity during end repair filled in the 5' overhangs, and single ‘A’ nucleotide was added to the 3’ ends of the blunt fragments to prevent ligations to one another during the adapter ligation reaction, thus ensuring low rate formation of chimeras, or concatenated templates. Next, multiple indexing adapters were ligated to the ends of cDNA. The libraries were amplified in 10 cycles of PCR in order to selectively enrich cDNA fragments that had adapter molecules on both ends, and to amplify the amount of cDNA in the library. Quantification of the cDNA libraries was performed through qPCR using the Kapa Illumina Library Quantification Kit (Illumina, San Diego, USA). The indexed cDNA libraries were normalized to

10nM using Tris-Cl in the Diluted Cluster Template (DCT) plate and then pooled in equal volumes in the Pooled DCT Plate (PDP) plate.

2.3.2 Transcriptome sequencing

HiSeq version 3 flow cell clustering was performed using TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, USA). Pooled samples were diluted to the final concentration of 12 pM and loaded onto the flow cell with a multiplex pool of 20 samples sequenced across 4 lanes. The single-read cluster generation was processed in rapid mode using the HiSeq 2500 (Illumina, San Diego, USA). Sequencing by synthesis of the libraries was performed on a HiSeq 2500 System with 100 paired-end cycles using TruSeq SBS Rapid – HS chemistry (Illumina, San Diego, USA). The raw FastQ sequences and demultiplexing of samples were obtained using Illumina Off-Line Basecaller (Illumina, San Diego, USA), and paired-end 100 base pairs (bp) read sequences were produced. Both cDNA library preparation and transcriptome sequencing were conducted at Edinburgh Genomics.

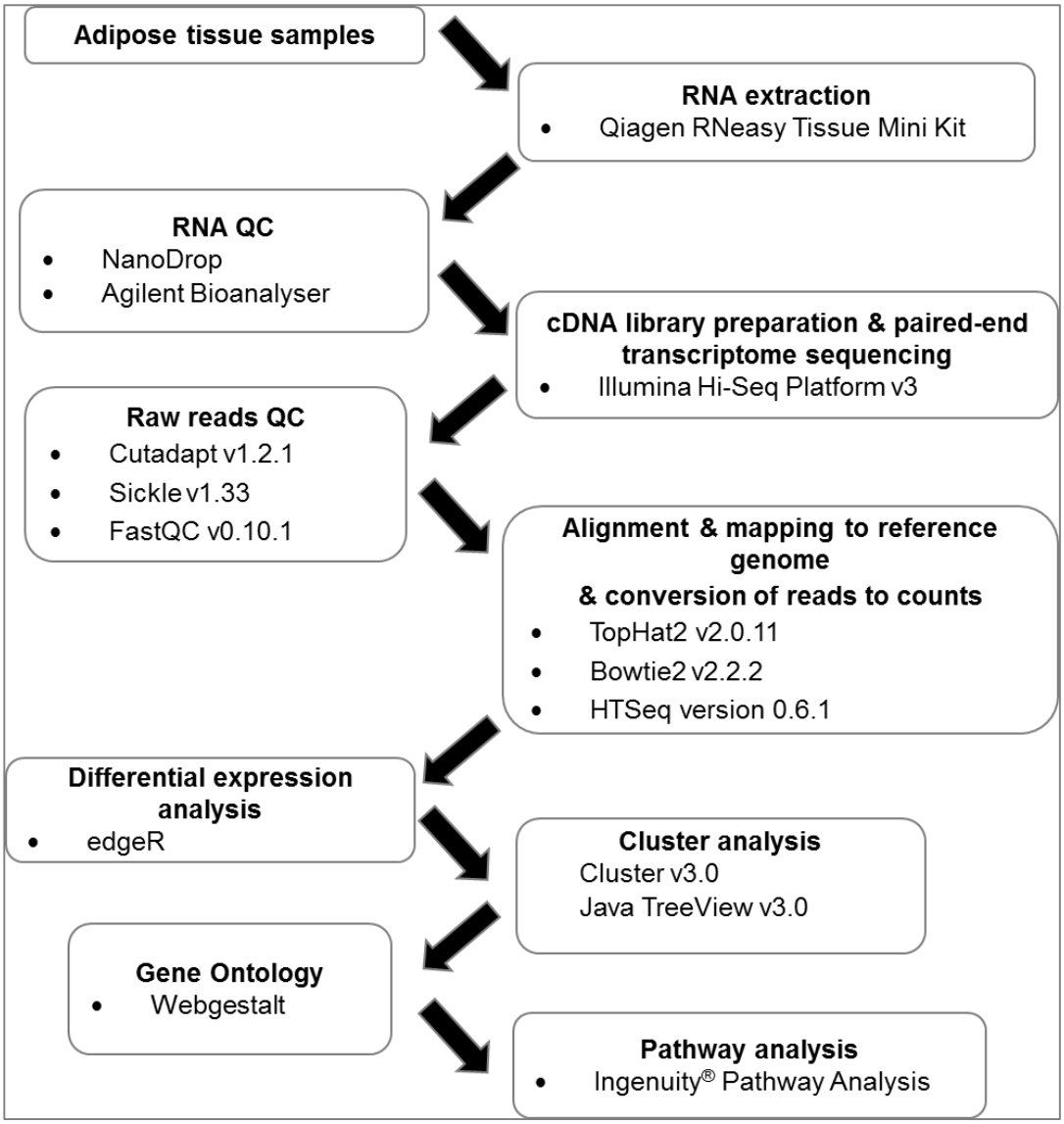


Figure 2.1 Flow diagram of RNA sequencing analysis. RNA was extracted from AT samples using Qiagen RNeasy Tissue Mini Kit, and its quality was assessed with NanoDrop and Agilent Bioanalyser. cDNA library preparation and paired-end transcriptome sequencing was performed on the Illumina Hi-Seq Platform, producing paired 100bp reads. Adapters were removed from the raw reads using Cutadapt, followed by QC with Sickle and FastQC. TopHat2 was used as a wrapper for the alignment programme Bowtie2 to map sequence reads to the reference genome, reads were converted into counts using HTSeq, and the output was used for differential expression analysis with edgeR. A heat map was generated using Cluster and Java TreeView to obtain a global view of gene expression across samples. Gene ontology was carried out using Webgestalt, and Ingenuity® Pathway Analysis was used to investigate interaction of genes identified in the dataset.

2.3.3 Quality control and alignment of transcript

Adapter sequences were removed from fastq files using Cutadapt version 1.2.1 (Martin, 2011). Sickle version 1.33 (https://github.com/ucdavis-bioinformatics/sickle) was employed to trim off poor quality bases, which resulted from the deteriorating quality of reads towards the 3′-end, as well as some towards the 5′-end that commonly occur in sequencing technologies (Joshi and Fass, 2011). Quality control of fastq files was crucial to eliminate high error sequences prior to mapping (Paszkiewicz and Studholme, 2010).The quality score was set at a phred score of 20, and unknown nucleotides (designated as N) were trimmed. Reads with less than 50 bases after trimming were discarded. This was to reduce the number of genes with low relative abundance. Quality control checks were completed on the raw sequence data using FastQC version 0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). A report was generated for each sample, covering quality of bases and sequences, content of bases, length of sequences, incidence of sequence duplication and over-representation.

Trimmed sequences were aligned to the latest genome assembly for the corresponding species at the time of analysis. BAM files of samples to be inferred in differential expression (DE) analysis were then merged into counts files using HTSeq version 0.6.1 (https://pypi.python.org/pypi/HTSeq); (Anders et al., 2014). Command lines used for this quality control and mapping process are listed in Appendix 1.

2.3.4 Differential gene expression analysis

Genes with significant evidence of DE were determined using edgeR version 2.4.6 (www.bioconductor.org/packages/release/bioc/html/edgeR.html); (Robinson et al., 2010). Command lines used in differentially expressed gene (DEG) analyses are listed on Appendix 1. The edgeR programme uses empirical Bayes estimation and exact tests based on the negative binomial distribution, with model-based scale normalization of sequence data, to identify statistically robust DEG, and this is reliable even for data of small sample sizes (Robinson and Oshlack, 2010, Robinson et al., 2010, Zhang et al., 2014b). Corresponding p-values were also calculated, and adjusted for false discovery rate (FDR). The level of significance used was of a fold change (FC) ≥ 2.0, and FDR < 0.05. Among the DEG identified, a number of Ensembl identifiers without corresponding official gene symbols were found. Thus further searches and conversion were required through The Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (Huang et al., 2009) and Ensembl Genome Browser (Flicek et al., 2013).

Three different plots were generated from the comparisons using edgeR: multidimensional scaling (MDS) plot, smear plot and volcano plot. A MDS plot illustrates the relative similarities or differences between the samples, by showing distances, in terms of the biological coefficient of variation (BCV), between samples. A smear plot displays the log-FC (y-axis) against average count size (mean-difference, x-axis), and DEG were highlighted on this plot. A volcano plot relates log-FC (x-axis) and negative log p-values (y-axis). The values that represent large magnitude FCs are displayed further left- or right-

of centre, and those of high statistical significance are towards the top of the plot. In addition to these plots, a plot showing the distribution of DEG by FDR over log FC was produced using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

2.4 Cluster analysis and gene ontology annotation

Unsupervised hierarchical cluster analysis was conducted on normalised count values of mapped genes using the GUI programme Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm); (de Hoon et al., 2004). Gene expression values were log transformed, and in order to reduce statistical noise to the resulting clusters, these were filtered to remove genes with insufficient read depth or intensity, as well as to remove samples that did not vary much. Criteria were set to remove all gene expression below 50 read counts, and to remove gene expression with FC < 2.0. Gene expression values were mean centred and average linkage hierarchical clustering was performed. Heat maps and dendrograms were generated with Java Treeview 3.0 (http://jtreeview.sourceforge. net/); (Saldanha, 2004) using a CDT file produced by an open source clustering software, Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm); (de Hoon et al., 2004). Pixel settings were changed to maximise the x- and y-axis, and the final image was exported as a PNG file.

Gene Ontology (GO) annotation analysis was performed using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt), a web-based analytic software (Wang et al., 2013). DEG lists were uploaded onto this platform and reports of

GO term enrichments were generated in categories which included biological processes (BP), molecular function (MF), and cellular component (CC). Prior to upload to WebGestalt, cattle, pig and elephant Ensembl identifiers were converted to orthologous human Ensemble identifiers due to limited annotation of these species. Genes significantly enriched within known signalling pathways were clustered, thus facilitating identification of biological pathway and functional processes that may be of interest.

2.6 Synthesis of cDNA and analysis by quantitative reverse transcription PCR

Validation of RNA-seq results was performed on all adipose samples using qRT-PCR. Reverse transcription (RT) of 1µg of total RNA into cDNA was performed using the qScript^{TM} cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, Maryland, USA). The 5X qScript Reaction Mix, and 50X qScript Reverse Transcriptase from the qScriptTM cDNA Synthesis Kit, as well as extracted RNA template were thawed on ice. Once thawed, each solution was mixed by gentle inversion, centrifuged briefly (9,632g for 10 seconds at 4^{o}C) to collect residual liquid from the sides of the tubes, and stored on ice.

The RT master mix for one reaction was prepared by adding 2µL of qScript Reaction Mix, and 0.5µL of qScript Reverse Transcriptase in a microcentrifuge tube. The master mix was gently mixed by inversion and placed on ice. Amount of reagents used was increased accordingly to the number of samples. A variable volume of RNA extracts amounting to 1µg RNA was added to 2.5µL RT master mix in a microcentrifuge tube, and the remaining

volume of RT master mix was made up with RNase-free water to reach a total of 10µL. The mixture was inverted gently, briefly centrifuged (9,632g for 10 seconds at 4^{o}C) and stored on ice. Samples added with RT master mix were then placed in room temperature for 5 minutes, followed by incubation at 37^{o}C in a water bath for 60 minutes. After incubation, cDNA was diluted with 190µL of RNase-free water and stored at -20^{o}C.

Quantification of gene expression via qRT-PCR was performed using TaqMan® Gene Expression Assays (Applied Biosystem, Carlsbad, CA, USA), with gene specific hydrolysis probe sets. A cocktail of reaction mix was prepared, consisting of 0.65µL of TaqMan® hydrolysis probe and primers, 7.5µL of Roche LightCycler 480 Probes Master (Roche, Burgess Hill, UK), and 4.85µL of diethylpyrocarbonate (DEPC) treated water, to make up 13µL total volume for a single PCR reaction, in a microcentrifuge tube. The reaction mix was pipetted into 96-welled PCR plates, and 2µL of cDNA templates were added in triplicates. The plate was run using LightCycler® 480 (Roche Diagnostics, Mannheim, Germany), and quantification of expression was carried out using LightCycler® 480 software. The relative PCR expression of genes of interest for samples was calculated in relative to expression of reference genes. The FC was further calculated using the Pfaffl method (Pfaffl, 2001).

2.5 Pathway analysis

The human orthologues of DEG lists were submitted to Ingenuity® Pathway Analysis (IPA); (http://www.ingenuity.com/products/ipa) for further investigation of the biological functions and pathways that were most significantly affected by multiple genes differentially expressed between the breeds in cattle and pigs, dietary protein levels in cattle, and sexes in African elephants, based on the Ingenuity® Knowledge Base (Gusev, 2008). Analyses were divided into sections: (i) Canonical Pathways (ii) Disease & Functions (iii) Networks, (iv) Upstream Regulator and (v) Regulator Effects.

Canonical Pathways Analysis identified pathways that were enriched for DEG from the dataset, and this was reported in the form of a bar chart. Significance of enrichment was determined through calculation of activation Z-scores that made predictions about potential regulators by using information about the direction of gene regulation, predicting either activation (shown as orange bars), or inhibition (in blue bars). A ratio line across the bar chart indicates the number of genes in the dataset over the total number of genes related to the particular pathway of the bar. Disease & Functions Analysis indicated pathologies and biological functions affected by DEG visualised as a TreeMap (hierarchical heat map) in boxes representing a family (or category) of related functions. Each rectangle represents a particular biological function or disease and the colour indicates its predicted state: orange for increased activities, and blue for decreased activities. Darker colours indicate higher absolute z-scores, and the size of the rectangles correlates with increased significance according to number of genes, -log p-values, or z-scores.

Networks showed interactions of genes from reported pathways in the literature. Upstream Regulator Analysis and Regulator Effects Analysis display molecules that regulate significant pathways, this helped to identify molecules upstream and downstream of the genes in the dataset that would potentially explain the observed expression changes, and also predicted whether those processes were increased or decreased. Results from Networks Analysis, Upstream Regulator Analysis and Regulator Effects Analysis were summarised in a table, showing related genes in enriched pathways, regulators, target genes, p-values, and z-score, and predicted activation through interactions of regulators and target genes (Kramer et al., 2014).

Further analysis could be achieved by overlaying different DEG lists, canonical pathways, and additional genes to build up pathway networks. Molecular Activity Predictors were used to predict activation or inhibition of molecules in pathways in reaction to DEG.

Chapter 3 Molecular Characterisation and Fatty Acid Profiling of Adipose Tissue of Cattle Breeds and Dietary Protein Levels

3.1 Introduction

3.1.1 Origin and domestication of cattle

Cattle are even-toed ungulates and members of the Artiodactyla order of mammals that belong within the Bovidae family, and the subfamily Bovinae. Existing domestic cattle breeds were descended from *Bos primigenius*, also known as Urus or Aurochs (King, 2004b), and are categorised into two taxa: the western cattle, *Bos taurus* (taurine) and the eastern cattle, *Bos indicus* (Zebu); (Loftus et al., 1999). The aurochs was a species of the northern hemisphere that evolved in Asia and later extended into Europe and Africa during the Pleistocene period giving rise to 3 different types: Asian, African and European (Porter, 1991b). Domestication of the aurochs is believed to have occurred approximately 9,000 years ago in the Near East (Troy et al., 2001). Through screening of microsatellite loci and mitochondrial genetic diversity from multiple breeds of western and eastern cattle, supported by archaeological data, it is believed that European taurine cattle originated in western Asia (Bradley et al., 1996, Loftus et al., 1999). Mitochondrial DNA sequence studies also suggested that European aurochs did not contribute to the gene pool of European modern cattle (Edwards et al., 2007). The Asian aurochs became extinct more than 2,500 years ago, followed by the African aurochs around 1,400 years ago. The death of the last female European aurochs in 1627 marked the extinction of this species (Porter, 1991b).

The domestication of cattle in Britain began in Neolithic times, around 2,000 BC. Over the centuries, occupation and settlement by Romans, Anglo-Saxons and Vikings brought in animals that may have contributed to the various regional types which later developed into the different breeds present today (Porter, 1991a). The Romans made the most profound impact through selective breeding to improve bloodlines of livestock and such knowledge was transferred to the farmers of Britain. After the decline and fall of the Roman Empire in the early fifth century, the next important milestone in cattle production occurred during the Agricultural and Industrial Revolution that started in the 18th century (King, 2004a). With almost threefold increase in the human population of London, the demand for meat and milk increased accordingly. However, city dwellers generally did not have access to land to produce their own food. This not only resulted in the need for better quality animals to produce meat and milk in larger quantities, the livestock industry also changed such that animal rearing was concentrated in the northern and western regions of the country (Porter, 1991a, King, 2004a). Animals were driven to slaughterhouses in London after being fattened in the Midlands, Norfolk, and other areas closer to London (Porter, 1991a, King, 2004a).

In the UK, cattle had originally been selected for draught work, while milk and meat production were secondary (King, 2004a). At the time, beef came from draught oxen that were no longer in prime working condition. However, horses began to replace working oxen as draught animals. Later, draught animals were reduced or eliminated altogether following the use of engines and machines. By the end of the 18th century, the function of cattle had shifted from being the beast of burden towards their use as a source of animal protein

(King, 2004a). Moreover, advances in farming practices enabled both increased fodder crop production and improved fodder storage practices, which enabled more animals to be fed, particularly in the winter. In turn, a greater number of animals produce more manure to fertilise crops and increase harvest (King, 2004a).

3.1.1.1 Development of cattle breeds

In the mid-1800s, breed societies were developed and herd books were kept for particular breeds to document stock and breeding records. This was done to ensure that the progeny possessed the desired traits of their sires or dams. Also, standards were established by breed societies to distinguish individual breeds, and to promote specific breeds. Once the desired characteristics were achieved, cross-breeding was discouraged, thereby establishing and maintaining the purebred status of registered animals (King, 2004a).

Ancestors of the most popular dairy breed, Holstein, originated from cattle raised in the northern region of Europe, with low and flat meadows, where dairy farming was evident from the 1st century during the Roman period (Edwards et al., 2011). The major historical development of the Holstein occurred in the Netherlands, more specifically in the two northern provinces of North Holland and Friesland. The original stock were black animals of Batavians, and the white animals of Friesians, that came with migrant European tribes who settled in the Rhine Delta region approximately 2,000 years ago. The cross-breeding of these animals resulted in the recognisable high-producing black-and-white dairy cows today (OSU, 2000b).

The beef breeds were developed from heavily muscled draught cattle possessing the attributes of fast growth and superior feed conversion, i.e. the ability to transform poorer quality grass into valuable animal protein (Porter, 1991c). The Hereford breed is believed to have descended from the small red cattle stock of Roman Britain, a large Welsh breed found along the English-Welsh border (Porter, 1991a, TheCattleSite, 2014). It was first developed in Herefordshire as a large stout, hardy draught animal, but later adopted a triple-purpose as a draught, meat and milk animal. However, it was soon recognized as a high producing beef animal (OSU, 2000a). The Hereford breed society was founded in 1878, while the herdbook was opened in 1846 and closed in 1884, ensuring the purity of the breed for over 130 years (Porter, 1991a). The origin stock of the Aberdeen Angus (AA) is believed to have started with black polled cattle found in Aberdeenshire, Scotland. During the mid-18th century, bulls were brought in from England, the Netherlands, and southern Scotland to improve these animals. By early 19th century, the type was gradually fixed as black and polled, having a short gestation period, early maturity and good beef marbling. The herdbook was established in 1862, which included the polled Galloway, and by 1867 the breed was officially known as the AA (Porter, 1991a).

3.1.1.2 Characteristics of Holstein, Hereford and Aberdeen Angus

The average British Holstein cow stands 145cm at the withers and weighs around 655kg, while the bull is up to 183cm in height and 1,360kg in weight (Porter, 1991a). The Holstein is the predominant breed for milk production in

the UK and many other countries in the world because of its ability to efficiently produce high volumes of milk (Holstein-UK, 2014). In 2010, the total daily milk yield from a Holstein cow in UK was reported to be about 7,330 litres/year (Holstein-UK, 2014). Compared to the Friesians, a smaller but dual-purpose breed for milk and beef, the conformation of Holsteins is poorer for beef, but because of its greater size it seemed to be able to produce a similar amount of meat, although of lesser quality (Porter, 1991a). Comparison of carcasses from Holstein, Angus and Hereford steers showed that Holsteins had the highest average daily weight gain, yet this was compensated with greater feed intake compared to the beef breeds, and its carcass had less trimmable fat but equal weight of trimmed prime cuts compared to AA and Hereford of the same live weight (Thonney, 1987). In another study, Holstein bulls attained the lowest percentage of kidney fat, while AA bulls were found to have the highest percentage of kidney fat (Holló et al., 2012). Meanwhile, Holstein cows had a higher deposition of visceral fat compared to beef breeds. However at times of negative energy balance (NEB), especially during the early lactation period, this depot was mobilised to meet energy demands, and continued to be depleted throughout the peak production period (Baldwin et al., 2004, Pfuhl et al., 2007). Thus, multiparous dairy cows would generally have a lower fat mass compared to beef breeds.

Male Herefords are commonly 150cm in height, and weigh 895 - 1,300kg, while females stand at 136cm and weigh 630 - 825kg. Herefords are compact, blocky, short-legged but fine-boned, fatten readily and mature early, thus making it an excellent beef producer (Porter, 1991a). Central performance testing organised by the Irish Cattle Breeding Federation (ICBF) reported

Herefords as having the highest milk production, fertility and calving rates, and lowest calving interval when compared to other beef breeds, such as AA, Simmental, Charolais, Limousin, Belgian Blue (Shirley, 2011). Its hardiness and placidity also facilitate management of the breed (Porter, 1991a).

An AA cow typically weighs around 550kg and the bull 850kg (RBST, 2015). Compared to continental beef breeds such as Simmental and Charolais, AA bulls record the highest fat scores with significantly higher intramuscular fat content of the longissimus muscle (Holló et al., 2012). The daily weight gain of AA in Britain were reported to average around 1.23kg per day, and performance-tested bulls achieved average 400-day weights of 460kg. Calves are small at birth, at 30 – 35kg, therefore reducing the risk of dystocia (Porter, 1991a). Results of central performance testing by ICBF showed that AA trailed very closely behind Hereford in terms of calving interval, milk production and fertility rate, and had lower new born mortality rate than Herefords (Shirley, 2011).

3.1.1.3 Economic value of beef and milk production and the challenges faced by the industry

The beef and dairy industries are important components of the UK economy. The value of UK beef and dairy exports increased from £331.7 million and £979.9 million respectively in 2010, to £462.1 million and £1.4 billion respectively in 2014 (FDF, 2012, FDF, 2015). Annual milk yields of dairy cows increased during the same period from 7,330 litres/animal to 7,916 litres/animal. Thus, dairy cows produced a total of 13,564 million litres of milk

in 2010, compared to 14,649 million litres in 2014 (FDF, 2012, FDF, 2015). However, beef production has decreased from 897,000 head in 2010, to 877,600 head in 2014 (EBLEX, 2015). Nevertheless, the monetary value of beef exports has continued to increase, despite the drop in production levels, due to the rise in beef prices and the higher currency exchange rate for British pounds. However the Department for Environment, Food & Rural Affairs (DEFRA) recorded a drop of 25.5% in the total UK cattle population from 1985 to 2014. The dairy breeding herd stabilised at 1.8 million head from 2010 – 2014. However, this is still a decline of more than 28% compared to the number recorded in 1996 (DEFRA, 2015a, EBLEX, 2015).

The decline in the cattle population was driven by numerous factors in the beef and dairy sectors. While milk production is focused solely on dairy animals, beef are produced from both the beef suckler herd and the dairy herd (EBLEX, 2008). In the 1990s, two thirds of beef cattle were sourced from the dairy herd. However this pattern was reversed when animals older than 30 months were banned from entering the human food chain in 1996 in response to an outbreak of Bovine Spongiform Encephalopathy (BSE). The BSE crisis not only reduced beef production, but also decreased consumer demand for beef (EBLEX, 2008). After the withdrawal of this ban in November 2005, a decade after the last significant BSE outbreak, supply of beef from the dairy herd again overtook the beef suckler herd, especially with the continuous decline of the UK's beef herd population, while the dairy herd stabilised (EBLEX, 2012). The challenge to the industry represented by a low profitability due to high production costs, was believed to be a major cause of the decline in the beef breeding herd. The economic sustainability of beef herds was further

challenged by cereal price inflation and the fact that beef retail prices have yet to recover to their level from 20 years ago (EBLEX, 2012). In addition to BSE, economic losses due to foot and mouth disease (FMD) outbreaks in 2001 and 2007 also resulted in severe loss of confidence among the beef producers (EBLEX, 2008).

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, is currently a major threat faced by both beef and dairy farmers in UK (NFU, 2014). The incidence of bTB has been in overall long-term upward trend in UK cattle herds since 1996, although the incidence rate is now lower than its peak in 2008 (DEFRA, 2015c). For the past ten years, an average of 32,455 heads of cattle tested positive or were in direct contact with reactors, and were slaughtered yearly (APHA, 2015). This impose a great economic impact on the farmers in terms of the loss of animals' value and their products, as well as on the government, which provides compensatory payments to the farmers. It was estimated that the average cost of a confirmed herd breakdown in high risk areas of England was £14,000 to the farmers and £20,000 to the government (DEFRA, 2015c). The risk of zoonotic transmission of bTB to human is relatively low in UK as commercially available milk is pasteurised, and infection through meat consumption is less likely as TB lesion generally do not appear in the meat. Infection may occur in workers handling infected animals or carcasses through inhalation of pathogenic materials (de la Rua-Domenech, 2006, Thoen et al., 2006). However, incidence of the *M. bovis* infection in humans is very low with an average of 29 cases annually, which accounted for only 0.57% of all culture confirmed human TB cases notified between 2004 to 2013 in UK (PHE, 2014).

The dairy cattle industry faces further significant challenges. The three major principal challenges faced by UK dairy farmers are (i) low fertility, (ii) mastitis and (iii) lameness. Low fertility is arguably the most significant challenge to the economical sustainability of the industry and it is also the most challenging to address. In addition, mastitis and lameness can contribute to low fertility (Royal et al., 2000). Improvement in milk production of dairy cows appeared to have led to a significant decline in fertility. This has been reported not only in UK, but in countries and regions that operate diverse production systems, such as North America, Ireland, New Zealand, and Australia (Royal et al., 2000, Lucy, 2001, Bousquet et al., 2004, Dillon et al., 2006, Macdonald et al., 2008). According to these reports, the culling of dairy animals was mainly due to poor fertility, which inevitably reduced their productive lifespan, lifetime milk yield, and number of offspring produced. Such economic losses have directly correlated with low profitability for the dairy industry (Ducrocq et al., 1998). Furthermore, the low number of offspring produced per cow would limit the availability of potential replacement heifers born within a herd, yet because of poor fertility within the herd, more heifers are needed to replaced culled animals (Wathes et al., 2008).

Fertility is a multi-factorial trait and its deterioration has been attributed to genetics, the environment, and the farm management systems used. These factors are dynamic and interact with one another, thus it is difficult to identify the exact causes of poor reproductive performances (Walsh et al., 2011). The current study focused on effects linking adipose tissue (AT) and reproductive performances, thus nutritional factors would be further elaborated as these could directly influence adiposity, fat metabolism, energy storage and

utilisation process in the animal. It has been proposed that inappropriate management of high producing dairy cows may have contributed significantly to the poor fertility of these animals, instead of the direct genetic effects (LeBlanc, 2010, Walsh et al., 2011). Also it has been suggested that better nutritional and reproductive management may result in high reproductive performance even in high producing dairy herds (LeBlanc, 2010, Walsh et al., 2011). Furthermore, higher energy and protein intake in Holstein heifers has been reported to result in an increased growth rate and earlier attainment of puberty (Lammers et al., 1999).

Both dairy and beef farmers typically seek to breed replacement heifers by 15 months of age so that animals calve for the first time at 24 months of age. This is widely perceived as the minimum age at which animals are sufficiently mature to calve without difficulty and to achieve a good first lactation milk yield (Wathes et al., 2014). In the study by Lammers and colleagues (1999), a higher numbers of heifers with accelerated growth attained puberty at 12 months. Thus, by the time heifers were bred at 15 months, these animals had completed multiple oestrous cycles and were more fertile than animals on first oestrus. However, the same study also found that accelerated prepubertal growth regimen decreased first lactation fat-corrected milk yield (Lammers et al., 1999).

Energy balance in dairy cows decreases during the periparturient period, in the last few weeks prior to calving (Ingvartsen and Andersen, 2000). This has primarily been attributed to a reduction in feed intake that may be caused by both anatomical and metabolic factors. During this period, the growing foetus

and uterus are compressing the rumen, thus limiting its capacity for food intake (Ingvartsen and Andersen, 2000). At the same time, there is an increased circulatory concentration of non-esterified fatty acids (NEFAs), glycerol and ketone bodies, due to mobilisation of AT to support the high energy demand of fetal growth and mammary gland development. These metabolic by products, and the high levels of estrogen present during pregnancy, may falsely indicate high nutrient status of the animal or increased adiposity of AT to the hypothalamus, resulting in signals to reduce food intake, as has been shown in cattle, human and rodents (Grummer, 1993, Suga et al., 1999, Ingvartsen and Andersen, 2000, Brown and Clegg, 2010, Paoli et al., 2015). Sex steroids such as estrogen has been observed to increase LEP secretion (Shimizu et al., 1997b), which supresses food intake in both human and animals as described earlier (Keisler et al., 1999, Klok et al., 2007). As plasma 17β-estradiol (E_2) and estrone concentrations of pregnant cows in the third trimester are significantly higher compared to during the first trimester (Patel et al., 1999, Pape-Zambito et al., 2008), and estrogen levels increased significantly approximately five days prior to parturition (Ingvartsen and Andersen, 2000), this may in turn increase LEP secretion and suppress food intake of cows in late pregnancy and especially during periparturient period (Ingvartsen and Andersen, 2000, Brown and Clegg, 2010). The dramatic increase of daily milk yield peaking between four and eight weeks postpartum exacerbates the decline in energy balance, and cows typically enter a NEB phase. During this time, feed intake is unable to meet the energy requirements, and mobilisation of body reserves occurs (Grummer, 2007). If NEB is prolonged beyond the first two months postpartum, this would result in a

number of negative health consequences for the cow, including an increased risk of metabolic diseases, such as acidosis and fatty liver syndrome, reduced immune functions, a predilection for developing mastitis and laminitis, and a reduction in subsequent fertility due to depressed follicular development and therefore a failure in resumption of regular oestrous cycle (Roche et al., 2009).

While high producing dairy cows may suffer from a decline in fertility, beef cattle genetically selected for high growth rates have not been reported to have compromised fertility (Archer et al., 1998). As will be outlined later, the molecular comparison of AT of the most common UK dairy and beef breeds (Holstein and Hereford) presented in this study, furthers the understanding of the differences in nutritional uptake, fat deposition, and resource mobilisation between these breeds, and may eventually help in formulation of strategies to improve fertility in dairy cows. Furthermore, the effects of different dietary proteins levels on adipogenesis and fat deposition were examined in AA crosses to identify optimal nutritional management strategies for UK beef herds.

3.1.2 Effects of protein levels on adipose tissue

As will be outlined later, in addition to comparison of AT transcriptomes between different cattle breeds, the effects of dietary protein intake levels on adipose deposition was studied. Intake of a higher amount of dietary protein (sometimes colloquially referred to as the Atkins diet) has been reported to reduce body weight and lipogenesis in people (Parker et al., 2002, Westerterp-Plantenga, 2003), and in rats (Stepien et al., 2011). This has been attributed

to an increase of energy expenditure due to elevated gluconeogenesis from protein, as this pathway of protein metabolism is energetically costly compared to carbohydrate metabolism (Mikkelsen et al., 2000, Veldhorst et al., 2009), as well as increasing postprandial thermogenesis, resulting in greater energy expenditure (Johnston et al., 2002). A high protein diet also induced a greater fat oxidation rate resulting in elevated production of the ketone body β-hydroxybutyrate (Veldhorst et al., 2010). Increased β-hydroxybutyrate concentrations are believed to depress appetite, therefore causing a reduction in food intake (Arase et al., 1988, Scharrer, 1999, Fisler et al., 1995, Meckling et al., 2002, Johnstone et al., 2008).

A substantial proportion of dietary protein ingested by cattle is degraded by ruminal microorganisms into peptides and amino acids, these are termed rumen degradable protein (RDP). The majority of RDP is deaminated in the rumen to form free ammonia, the amino acids are then used to form microbial protein, and are thus incorporated into ruminal microbes. Ammonia can be absorbed into the bloodstream across the rumen wall and transported to the liver and synthesised into urea. Urea will either be excreted through urine, pass into saliva and return to the rumen, or be absorbed into the bloodstream and transported back to the intestine. On the other hand, rumen undegradable protein (RUP) can bypass ruminal degradation and reach the small intestine to be digested and readily absorbed as amino acids and peptides into the blood circulation (Cecava, 1995a, Cecava, 1995b). In addition to preformed protein, non-protein nitrogen (NPN) substances such as urea can also be present in feed. Urea can be converted into ammonia within the rumen, and be taken up by microbes to form microbial protein, as the majority of rumen

bacterial growth is heavily dependent on ammonia as its sole nitrogen source. It has been estimated that, in ruminants, microbial protein may contribute up to 50% or more of the animal's required protein. Thus, the amino acids absorbed and utilised by ruminants, may not be a direct reflection of their proportions in the feedstuffs they have ingested (Cecava, 1995b).

It is important to determine whether alterations of dietary protein levels affect AT in cattle, as this has been reported in humans, rats and pigs (Parker et al., 2002, Westerterp-Plantenga, 2003, Stepien et al., 2011, Madeira et al., 2013). However the complex digestive system of ruminants may respond differently to a change in dietary protein levels as compared to monogastric animals. Moreover, the site of *de novo* fatty acid (FA) synthesis in cattle was reported to be concentrated within the AT, as opposed to being in the liver of humans and in both tissues of rodents (Vernon, 2005). Because protein is utilised as the building blocks of all organs and systems, as well as the synthesis of hormones and enzymes (Cecava, 1995a), protein feed trial was performed in this study to determine if dietary protein in cattle could be modified to influence the nutritional requirement of the animals to provide optimal health for the animal and sustain high level of meat and milk production.

3.1.3 Fatty acid structure, composition and importance in ruminants

Material for *de novo* FA biosynthesis in ruminants is mainly derived from hydrolysis of (i) dietary esterified lipids to free fatty acids (FFAs) and (ii) glycerol by ruminal microbial lipases. This is followed by biohydrogenation, elongation and desaturation, and isomeric changes to yield FA of different

chain lengths and biochemical properties, although the main end products are saturated fatty acids (SFAs): palmitic and stearic acid (van Houtert, 1993, Jenkins, 1993, Mattos et al., 2000, Chilliard et al., 2001, Lock and Bauman, 2004, Jenkins et al., 2009). As a result, ruminant tissues rich in lipid such as meat and milk, usually contained high SFA levels, compared to chicken or pork (Hocquette and Bauchart, 1999, Lourenco et al., 2010). However, some FAs escape biohydrogenation after the initial lipolysis process, short-chain fatty acids (SCFAs) such as the volatile fatty acids (VFAs) can be directly absorbed through rumen wall into the bloodstream and transported to various organs, although most of the VFAs produced are being utilised by rumen microbes (Hocquette and Bauchart, 1999, Lourenco et al., 2010). Meanwhile, FAs with longer chain length are incorporated into chylomicron and transported to the duodenum, absorbed into the blood circulation, and are then being taken up by the AT, the mammary gland, and other tissues (Hocquette and Bauchart, 1999, Palmquist, 2006, Laliotis et al., 2010). On the other hand, linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) are the principal essential unsaturated FAs that need to be acquired exogenously. These FAs can then be elongated and desaturated into their longer-chain derivatives such as arachidonic acid (20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3); (Simopoulos, 1991).

The FA composition of AT is an important factor in milk and meat production in cattle as essential fatty acids (EFAs) stored in AT, besides from dietary sources, are mobilised for tissue growth and development thus crucial in maintaining various physiological functions throughout the body system (Simopoulos, 1999). In reference to reproduction, EFAs are precursors for

production of steroid hormones and prostaglandin, and polyunsaturated fatty acids (PUFAs) composition has been described to affect quality of oocytes and ovarian steroid synthesis, particularly progesterone (P_4); (Wathes et al., 2007). All these can influence conception rate and survival of conceptus throughout gestation. Depending on energy balance status of the animal, body fat reserve of the lactating cow contributed between five to over twenty percent of milk FAs (Lock and Bauman, 2004). Therefore FA composition of AT may reflect lipid metabolism in dairy cows and further the understanding of fat mobilisation from AT to meet the energy demand for milk production, as indicated in studies in human (Martin et al., 1991, Martin et al., 1993).

Higher dietary protein had been shown to reduce fat deposition, and research had also revealed that dietary protein levels affect FA composition in AT of cattle and pigs, however results from these studies did not show consistent changes in FA composition relative to dietary protein levels (Smet et al., 2000, Ward et al., 2010, Madeira et al., 2013). Therefore, it would be of interest to examine the effect of both breeds and dietary protein level on FA composition of AT, integrated with RNA sequencing (RNA-seq) results, as it was reported that breed could be a factor due to differences in lipogenic genes expression (Dance et al., 2009, Madeira et al., 2014), particularly of enzymes related to FA biosynthesis, such as fatty acid synthase (FASN), and stearoyl-CoA desaturase (SCD), that would be indicative of lipid metabolism in AT (Smith, 1994, Kim and Spiegelman, 1996, Kersten, 2001, Smith et al., 2003, Chirala and Wakil, 2004, Strable and Ntambi, 2010, Li and Cheng, 2014).

3.2 Materials and methods

3.2.1 Animals and samples

Peri-renal adipose samples were collected from ethically approved abattoirs shortly after the animal was humanely slaughtered. Samples were immersed in RNAlater® (Ambion (Europe) Ltd., Huntingdon, UK) then stored at -80ºC pending analysis. Holstein, Hereford, and AA were *Bos taurus* breeds selected in this this study as they are representative of the most important breeds widely used in the UK beef and dairy industries. Two adipose samples from each breed and dietary protein treatment were sequenced and subjected to RNA-seq analysis (Table 3.8 & 3.12), and transcriptomic results were compared.

In addition, adipose samples from Jersey (dairy breed), Highland, Charolais and Limousin (beef breeds) females were sequenced as these were other popular breeds in UK. However, as only one sample was collected for each of these breeds during this study, further analysis on these samples was not performed. The outline of sample processing and RNA-seq data analysis workflow was as illustrated in Chapter 2. Experimental protocols and operative procedures were reviewed and approved by the Research Directorate and the Ethics Committee at the School of Veterinary Medicine and Science, University of Nottingham.

3.2.2 Dietary protein feed trial

Aberdeen Angus crosses (AAX) heifers were used for dietary protein feed trial to examine the effects of dietary protein levels on AT. The AA cattle were

selected for this study as it is the most popular British beef breed in UK (NFU, 2013), therefore results from this study would be relevant to the beef industry. Animals were maintained on either low protein (10%) or high protein (14%) for a period of 8 weeks prior to slaughter. The low protein diet consisted of maize silage, grass silage, bread, oat feed, citrus pulp, fruits, chopped straw, lime flour, beef minerals, and yeast. The high protein diet was composed of grass silage, apple pulp, Poundon Pre-Mix, and feed grade urea. The nutritional content of the diets are shown in Table 3.1. The diets were designed to be isocalorific, and adjustment on the quantity of starch and sugar was done in order to compensate for the difference of energy content of the feed due to the changes in protein levels. In order to facilitate discussion later, LP and HP are therein defined as samples from animals on low or high protein diet respectively.

Table 3.1. Nutritional composition of low and high protein diets. The diets were designed to be isocalorific by adjusting mainly the quantity of starch and sugar to compensate for the difference of energy due to changes in protein levels.

	Low protein (g/kg DM)		High protein (g/kg DM)	
	Whole food	**Concentrates**	**Whole food**	**Concentrates**
Metabolisable energy (MJ/kg DM)	11.8	12.1	11.2	12.3
Crude protein	104.0	118	145.0	174
Crude oil	41.4	-	40.9	-
Neutral detergent fibre	320.0	-	395.0	-
Starch	302.0	-	145.0	-
Sugars	59.0	-	113.0	-
Long roughage (%)	49.2	-	49.3	-

DM = dry matter, Starch = polysaccharides, Sugars = mono- & disaccharides.

3.2.3 RNA-sequencing and Differential Expression Analysis

A total of 4 female (Holstein n = 2, Hereford n = 2) adult adipose samples were analysed (Table 3.8) to determine the effects of breed on AT metabolism. For comparison of effects of low (LP) and high dietary protein (HP) on AT in the AAX, 2 samples from each treatment group (LP n = 2, HP n = 2) were analysed (Table 3.12). Work flow and protocols of total RNA extraction, RNA-seq, GO, pathway analysis, and validation of RNA-seq through quantitative reverse transcription PCR (qRT-PCR) were as described in Chapter 2. Quality and adapter trimmed sequences for the cattle adipose samples were aligned to the *Bos taurus* genome assembly released in December 2009 (Btaurus UMD3.1.71, September 2011). The genome assembly encoded 19,994 coding genes, 3,825 non-coding gene, 3,650 small non-coding genes, 175 miscellaneous non-coding genes, 797 pseudogenes, and 26,740 transcripts (Ensembl, 2015a).

3.2.4 Quantitative reverse transcription PCR validation of RNA-sequencing results

Validation of RNA-seq results was performed on peri-renal adipose samples for comparison between breeds, and between LP and HP (Holstein n = 6, Hereford n = 6, LP n = 6, HP n = 6); (Table 3.2). The process of cDNA synthesis was as described in Chapter 2.6, using the the qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, Maryland, USA). The Taqman® Gene Expression Assays hydrolysis probesets (Applied Biosystem, Carlsbad, CA, USA) used are listed in Table 3.3.

Table 3.2 List of animals used for qRT-PCR validation of RNA-seq.

ID	Breed/ Protein levels	Age	Slaughter date
PR621C	Holstein	3y 8m	27 Feb 2013
PR655C	Holstein	6y 0m	27 Feb 2013
PR676C	Holstein	3y 4m	27 Feb 2013
PR697C	Holstein	4y 6m	27 Feb 2013
PR732C	Holstein	3y 0m	27 Feb 2013
PR752C	Holstein	4y 10m	27 Feb 2013
PR7667C	Hereford	1y 11m	4 Jun 2013
PR7668C	Hereford	1y 11m	4 Jun 2013
PR7669C	Hereford	1y 11m	4 Jun 2013
PR7887C	Hereford	2y 1m	4 Jun 2013
PR7888C	Hereford	2y 1m	4 Jun 2013
PR7889C	Hereford	2y 1m	4 Jun 2013
LP-PR39C	LP	2y 4m	30 Jan 2014
LP-PR40C	LP	2y 5m	30 Jan 2014
LP-PR41C	LP	2y 4m	30 Jan 2014
LP-PR1C	LP	2y 5m	13 Feb 2014
LP-PR4C	LP	2y 4m	13 Feb 2014
LP-PR6C	LP	2y 4m	13 Feb 2014
HP-PR2C	HP	2y 0m	9 Apr 2014
HP-PR3C	HP	1y 9m	9 Apr 2014
HP-PR5C	HP	1y 10m	9 Apr 2014
HP-PR12C	HP	2y 1m	29 May 2014
HP-PR13C	HP	1y 11m	29 May 2014
HP-PR14C	HP	2y 1m	29 May 2014

Note: y = years, m = months

Table 3.3. List of Taqman® hydrolysis probesets used for qRT-PCR. TBP and UXT were used as internal control.

Gene	Taqman® hydrolysis probeset ID
ASS1	Bt03230903_m1
CA2	Bt03224622_m1
ELOVL5	Bt03235952_m1
ELOVL6	Bt00907566_m1
FASN	Bt03210478_g1
IGFBP5	Bt03258786_m1
INSIG1	Bt03252733_m1
IRF4	Bt04290163_m1
LDLR	Bt04301299_m1
LEPR	Bt03210414_m1
NNAT	Bt03224018_m1
PER2	Bt04311405_m1
RAN	Bt03227534_g1
RXRA	Hs01067636_m1
SCD	Bt04307476_m1
TBP	Bt03241947_m1
UXT	Bt03229278_m1

3.2.5 Determination of adipose tissue fatty acid composition

The lipid component of AT was extracted from peri-renal AT (Holstein n = 6, Hereford n = 6, LP n = 6, HP n = 6); (Table 3.4) as previously described (Nilsson et al., 2014). The FA extraction was carried out in the fume hood. A total of 500mg AT were homogenised in 2mL of 2:1 chloroform-methanol solution with 1mL of QIAzol Lysis Reagent (Qiagen, Crawley West Sussex, UK) using a gentleMACS M tube (VWR International Ltd, Leicestershire, UK) at 1,000rpm for 90 seconds on the gentleMACS dissociator (VWR International Ltd, Leicestershire, UK). Homogenised samples were agitated for 15 - 20 minutes at room temperature using the Stuart gyro-rocker (Bibby Scientific Limited, Stone Staffordshire, UK). The homogenate was filtered into a 50mL Falcon conical centrifuge tubes using Whatman Grade 1 Qualitative Filter Paper Standard Grade (GE Healthcare UK Ltd., Buckinghamshire, UK). An additional 8mL of 2:1 chloroform-methanol solution (Chloroform: Sigma-Aldrich, Dorset, UK, methanol: Fisher Scientific, Loughborough, UK) was used to rinse the M tubes, and this solution was also filtered. This was followed by adding 3mL of 0.9% sodium chloride (Fisher Scientific, Loughborough, UK) solution to the filtered homogenate.

Samples were vortexed (Evolution ZX FB15012 TopMix, Fisher Scientific, Leicestershire, UK) then centrifuged (Heraeus PICO 17 Centrifuge, Thermo Scientific, Osterode, Germany) at 555g for 10 minutes at room temperature. The upper lipid layer was aspirated into a microcentrifuge tube, and samples were vortexed and centrifuged again to retrieve any residual lipids. A volume of 1mL extracted lipid was dried under a nitrogen stream (Techne Sample

Concentrator FDB03DD, Techne Duxford, Cambridge, UK) for approximately 30 minutes. Samples were then re-suspended in 2mL hexane (Fisher Scientific, Loughborough, UK). However if the methylation process was not to be carried out on the same day, only 1mL hexane was used, and samples were stored at -20^{o}C freezer pending methylation. Prior to methylation process, the frozen samples should be thawed, and 1mL hexane was added to each sample and vortexed for 5 seconds.

Methylation of samples was initiated with 40µL methyl acetate, and 40µL methylation reagent was added into each sample tube. The mixture was vortexed for one minute then left to stand for 10 minutes. Subsequently, 60µL termination reagent was added, and vortexed for 30 seconds. Approximately 200mg calcium chloride (Fisher Scientific, Loughborough, UK) was then added and samples were vortexed, and left to stand for an hour. Samples were then centrifuged at 1,113g for five minutes at 15°C. Samples were transferred into individual crimp vials, and 10µL of C19 (Sigma-Aldrich Co LLC, Gillingham, UK) was added to each as internal control. Crimp vials were sealed with metal crimp lid and electronic crimper (Agilent Electronic Crimper 5183-4263, Santa Clara, CA, USA) and stored at -20^{o}C pending profile analysis.

Methylation reagent and termination reagent were only mixed on the day that the methylation process was to be performed, as these reagents have a short shelf life once prepared. The methylation reagent comprised of 0.9mL 30% sodium methoxide (Sigma-Aldrich, Dorset, UK) and 4.1mL HPLC grade methanol (Fisher Scientific, Loughborough, UK) and the termination reagent

contained 0.1g oxalic acid (Sigma-Aldrich, Dorset, UK) and 3mL diethyl ether (Sigma-Aldrich, Dorset, UK). Oxalic acid was dried in an oven at 95°C for 30 minutes prior to mixing with diethyl ether.

The relative quantity of FAs in each sample was determined using gas chromatography (GC), through injection of FA methyl esters at ratio of 50:1 into a gas chromatograph (GC 6890, Agilent technologies Ltd, Stockport, UK). Separation of FA methyl esters was performed with a Varian CP-Sil 88 (Crawford Scientific^{TM} Ltd., Strathaven, UK) capillary column with hydrogen as the carrier gas. Oven temperature was programmed from 59^{o}C to 100^{o}C at 8^{o}C per minute, then to 170^{o}C at 6^{o}C per minute and held for 10 minutes, and then to 240^{o}C at 3^{o}C per minute and held for 10 minutes. The temperature of the injector and detector were set at 255^{o}C and 250^{o}C respectively. The FA methyl esters were identified by comparing the retention times with a FA methyl esters standard mixture (Sigma-Aldrich Co LLC, Gillingham, UK) and the area percentage in moles were used for the statistical analysis. A total of 36 FAs were analysed in this study, including C19 as internal control. The SFAs, monounsaturated fatty acids (MUFAs) and PUFAs analysed are listed in Table 3.5, 3.6 and 3.7. The GC procedure was carried at the School of Biosciences, University of Nottingham.

The relative abundance of each FA detected was calculated by comparing individual readings with the total, and only FA with values above 0.1% were used for analysis. Differences between samples for each analyte were tested by ANOVA corrected by Bonferroni method using GraphPad Prism (version

6.05 for Windows) with false discovery rate (FDR) < 0.05 considered significant.

During the initial analysis of the AT's FA profiles were compared in relative to the breeds or dietary protein levels. The carcass conformation, fat class and dead weight of carcasses from the dietary protein trial were taken into account to further analyse the FA profiles.

Table 3.4 List of animals used for FA composition analysis using gas chromatography.

ID	Breed/ Protein levels	Age	Slaughter date
PR621C	Holstein	3y 8m	27 Feb 2013
PR676C	Holstein	3y 4m	27 Feb 2013
PR697C	Holstein	4y 6m	27 Feb 2013
PR732C	Holstein	3y 0m	27 Feb 2013
PR756C	Holstein	4y 10m	27 Feb 2013
PR348C	Holstein	4y 9m	26 Mar 2013
PR7667C	Hereford	1y 11m	4 Jun 2013
PR7668C	Hereford	1y 11m	4 Jun 2013
PR7669C	Hereford	1y 11m	4 Jun 2013
PR7887C	Hereford	2y 1m	4 Jun 2013
PR7888C	Hereford	2y 1m	4 Jun 2013
PR7889C	Hereford	2y 1m	4 Jun 2013
LP-PR39C	LP	2y 4m	30 Jan 2014
LP-PR41C	LP	2y 4m	30 Jan 2014
LP-PR4C	LP	2y 4m	13 Feb 2014
LP-PR6C	LP	2y 4m	13 Feb 2014
LP-PR8C	LP	2y 4m	13 Feb 2014
LP-PR11C	LP	2y 2m	13 Feb 2014
HP-PR4C	HP	2y 4m	9 Apr 2014
HP-PR6C	HP	2y 4m	9 Apr 2014
HP-PR7C	HP	2y 4m	9 Apr 2014
HP-PR12C	HP	2y 1m	29 May 2014
HP-PR14C	HP	2y 1m	29 May 2014
HP-PR15C	HP	2y 4m	29 May 2014

Note: y = years, m = months

Table 3.5. List of saturated fatty acids detected using gas chromatography.

Lipid number	Common name	Structural formula
C6:0	Caproic acid	$CH_3(CH_2)_4COOH$
C8:0	Caprylic acid	$CH_3(CH_2)_6COOH$
C10:0	Capric acid	$CH_3(CH_2)_8COOH$
C11:0	Undecylic acid	$CH_3(CH_2)_9COOH$
C12:0	Lauric acid	$CH_3(CH_2)_{10}COOH$
C13:0	Tridecylic acid	$CH_3(CH_2)_{11}COOH$
C14:0	Myristic acid	$CH_3(CH_2)_{12}COOH$
C15:0	Pentadecylic acid	$CH_3(CH_2)_{13}COOH$
C16:0	Palmitic acid	$CH_3(CH_2)_{14}COOH$
C17:0	Margaric acid	$CH_3(CH_2)_{15}COOH$
C18:0	Stearic acid	$CH_3(CH_2)_{16}COOH$
C20:0	Arachidic acid	$CH_3(CH_2)_{17}COOH$
C21:0	Heneicosylic acid	$CH_3(CH_2)_{19}COOH$
C22:0	Behenic acid	$CH_3(CH_2)_{20}COOH$
C23:0	Tricosylic acid	$CH_3(CH_2)_{21}COOH$

Table 3.6. List of monounsaturated fatty acids detected using gas chromatography.

Lipid number	Common name	ω-n	Structural formula
C14:1	Myristoleic Acid	ω-9	$CH_3(CH_2)_7CH=CH(CH_2)_3COOH$
C16:1	Palmitoleic acid	ω-7	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
C15:1	Pentadecenoic acid	ω-9	$CH_3(CH_2)_7CH=CH(CH_2)_4COOH$
C17:1	Heptadecenoic acid	ω-8	$CH_3(CH_2)_6CH=CH(CH_2)_7COOH$
C18:1n9t	Elaidic acid	ω-9	$CH_3(CH_2)7CH=CH(CH_2)_7COOH$
C18:1n9c	Oleic acid	ω-9	$CH_3(CH_2)7CH=CH(CH_2)_7COOH$
C20:1	Paullinic acid	ω-7	$CH_3(CH_2)5CH=CH(CH_2)_{11}COOH$
C22:1n9	Erucic acid	ω-9	$CH_3(CH_2)7CH=CH(CH_2)_{11}COOH$
C24:1	Nervonic acid	ω-9	$CH_3(CH_2)7CH=CH(CH_2)_{13}COOH$

Table 3.7. List of polyunsaturated fatty acids detected using gas chromatography.

Lipid number	Common name	ω-n	Structural formula
C18:2n6t	Linolelaidic acid	ω-6	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
C18:2n6c	Linoleic acid	ω-6	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
C18:3n3	Linolenic acid	ω-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$
C18:3n6	γ-Linolenic acid	ω-6	$CH_3(CH_2)_4CH=CHCH2CH=CHCH_2CH=CH(CH_2)_4COOH$
C20:2	Eicosadienoic acid	ω-6	$CH_3(CH_2)_4CH=CHCH_2=CHCH_2=(CH2)_8COOH$
C20:3n3	Eicosatrienoic acid	ω-3	$CH_3(CH_2)_4CH=CHCH_2=CH=CHCH_2=(CH_2)_8COOH$
C20:3n6	Dihomo-γ-linolenic acid	ω-6	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_6COOH$
C20:4n6	Arachidonic acid	ω-6	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
C20:5n3	Eicosapentaenoic acid	ω-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
C22:2	Docosadienoic acid	ω-6	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_{11}COOH$
C22:6n3	Docosahexaenoic acid	ω-3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$

3.3 Results

3.3.1 Transcriptomic analysis

3.3.1.1 Holstein versus Hereford

3.3.1.1.1 Statistics and read annotations

The Illumina Hi-Seq platform was used to generate paired-end sequence reads for RNA-seq analysis. Table 3.8 and Figure 3.1 show the number of raw reads obtained, and the breakdown of read numbers after quality control and mapping processes. The Hereford samples, HER1 and HER2, had a lower number of read counts as these were sequenced once, whereas Holstein samples, HOL1 and HOL2 were sequenced twice to generate sufficient reads

as specified in the original contract with Edinburgh Genomics. Raw reads generated from both sequences were pooled and normalised, thus higher read number in HOL1 and HOL2 would not result in greater mRNA expression levels when compared with sequences from Herefords. The percentages of aligned and QC reads were similar in all samples (Figure 3.2). A total of 11,902 of genes were annotated to *Bos taurus* genome in Ensembl database from the mapped reads, and these were used for subsequent analyses.

Table 3.8. Breakdown of RNA-seq reads for adipose samples of Holsteins and Herefords. Number of raw reads generated from RNA-seq are shown as total reads. QC reads are sequence reads kept after QC, and the ones that did not pass the QC processes are the discarded reads. Sequence reads that were confidently mapped to reference genome are presented as mapped reads, leaving the ones that were not mapped as unmapped reads.

ID	Breed	Age	Total reads	QC reads	Mapped reads	Unmapped reads	Discarded reads
HOL1	Holstein	3y	73,222,082 (100%)	72,268,540 (98.69%)	64,410,466 (87.97%)	7,858,074 (10.73%)	953,542 (1.30%)
HOL2	Holstein	7y	80,156,542 (100%)	78,156,542 (97.50%)	72,358,012 (90.27%)	5,798,530 (7.23%)	1,999,980 (2.50%)
HER1	Hereford	2y 7m	57,436,770 (100%)	55,443,166 (96.52%)	52,079,918 (90.67%)	3,363,248 (5.86%)	1,993,604 (3.47%)
HER2	Hereford	2y 10m	56,318,570 (100%)	54,558,446 (96.87%)	51,760,063 (91.91%)	2,798,383 (4.97%)	1,760,124 (3.12%)

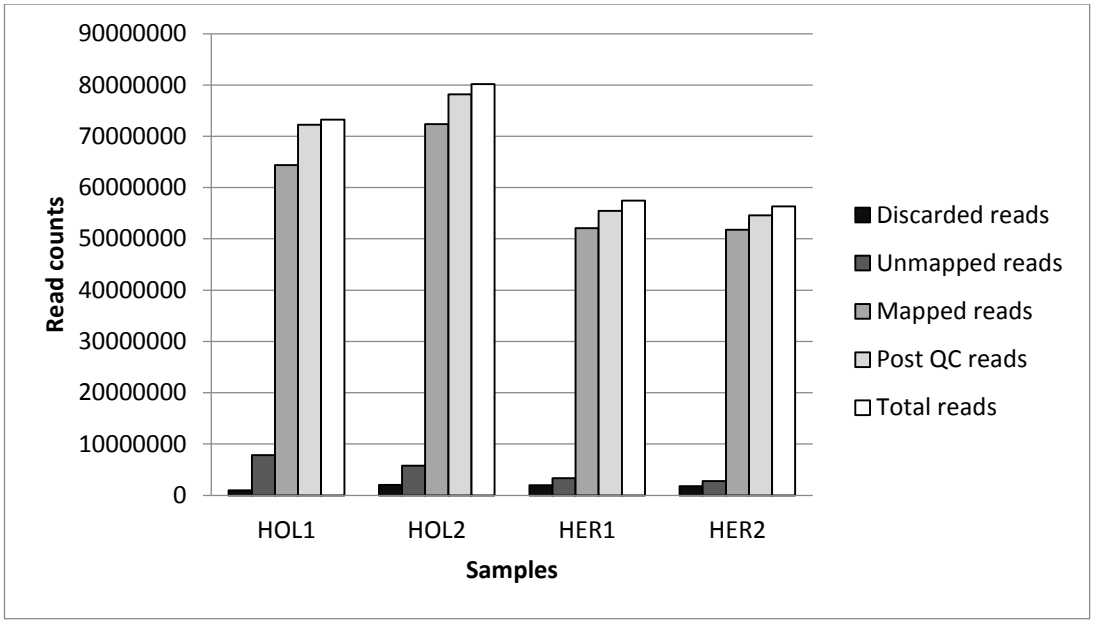


Figure 3.1. RNA-seq reads dispersions by count numbers for adipose samples of Holsteins and Herefords. Total reads refer to the number of raw reads generated from RNA-seq. Post QC reads are sequence reads that passed QC criteria, and the discarded reads were the ones that failed to pass QC. Mapped reads are sequence reads confidently mapped to reference genome, while the ones that were not mapped are presented as unmapped reads. HER1 and HER2 had a lower number of read counts as these were sequenced once, whereas HOL1 and HOL2 were sequenced twice.

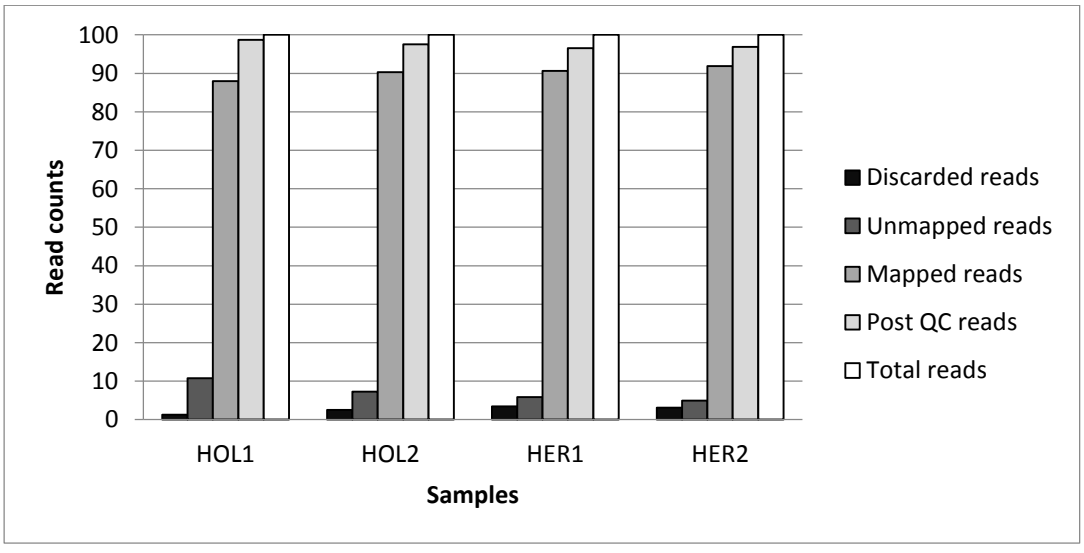


Figure 3.2. RNA-seq reads dispersions by percentages of count numbers for adipose samples of Holsteins and Herefords. This graph shows the breakdown of sequence reads in percentages corresponding to count numbers presented in Figure 3.1. Compositions of both Hereford samples appeared comparable with Holstein samples although the count number was lower.

3.3.1.1.2 Differentially expressed genes

The expression of 311 genes was found to be significantly different (FDR < 0.05) between peri-renal AT of Holsteins and Herefords based on differential expression (DE) analysis using edgeR, with cut off criteria set at fold change (FC) $\geq$ 2. Among these, 179 were more highly expressed in Herefords compared to Holsteins, while 132 gene expressions were higher in Holsteins relative to Herefords (Appendix 2). Filtering the gene lists for genes with a FC $\geq$ 2 and FDR < 0.05, narrowed down the pool of genes of interest for subsequent analysis (Figures 3.3, 3.4 and 3.5); (Benjamini and Hochberg, 1995).

From the list of top 25 genes with expression levels lower in Holsteins compared Herefords (Table 3.9), more than half of the genes were related to cell differentiation and proliferation with particular relevance to the musculoskeletal and nervous system according to information deposited in National Center for Biotechnology Information (NCBI) Reference Sequence Database, RefSeq (http://www.ncbi.nlm.nih.gov/refseq/): *TNC*, *IQGAP3*, *CDC20, EN1*, *SLITRK4*, *ACTG2*, *DES*, *MBNL3* (Sethi et al., 1991, Zec et al., 1997, Aruga and Mikoshiba, 2003, Irintchev et al., 2005, Wang et al., 2007, Holt et al., 2009, Atcheson et al., 2011, Lehtonen et al., 2012, Koutakis et al., 2015). Four genes were of particular interest as they have been implicated in lipid metabolism: *C19orf80*, *ASIP*, *ACLY*, *INSIG1* (Li et al., 2002, Zemel, 2003, Liu et al., 2012b, Migita et al., 2014, Barja-Fernandez et al., 2015). Two genes were associated with reproductive functions: *SPP1* was indicated to influence implantation and placentation in humans and animals, also variants of this

gene affects milk yield and composition in dairy cows (Cohen-Zinder et al., 2005, Fernandez-Rodriguez et al., 2011, Hernandez et al., 2013, Dudemaine et al., 2014, Hernandez et al., 2014); *S100A8* serum protein was elevated in women with early pregnancy loss (Nair et al., 2015). Collectively, GO was suggestive of enhanced muscular and AT development in the Hereford beef breed as compared to the dairy Holstein breed.

Meanwhile, among the top 25 genes with higher expression in Holsteins compared to Herefords (Table 3.10), a number of genes were involved in absorption, synthesis, release, and transport of nutritional elements including *RYR3* in intra-cellular calcium release (Baran and Ganea, 2013), *GIF* in vitamin B12 absorption (Tanner et al., 2005), *RDH16* in all-*trans* retinoic acid (ATRA) biosynthesis (Cain et al., 2002), *SLC16A12* in monocarboxylic acid and creatine transport (Halestrap and Meredith, 2004, Abplanalp et al., 2013), *FABP7* in FA uptake, transport, and metabolism (Glatz et al., 2001), and *GFPT2* in glucose utilisation and energy metabolism to generating energy (in the form of ATP) from nutrients (Zhang et al., 2004, Prasad et al., 2010). Other genes were implicated in immune reactions: *TNFAIP6*, *IRF4*, *SLAMF9*, *IL1RL1* (Calpe et al., 2008, Eguchi et al., 2008, Eguchi et al., 2011, Lauer et al., 2015, Traister et al., 2015); cell proliferation: *HES6*, *PPP2R2B*, *NOX4*, *GRB7* (Haapa-Paananen et al., 2012, Fang et al., 2013, Schroder et al., 2009, Lim et al., 2014), and reproductive organ functions: *OOSP1* and *AKAP4* (Hu et al., 2009, Tremblay et al., 2006). The expression of these genes may be reflective of the physiological demands of high milk production in the Holsteins, compared to the requirement for muscular development in the Herefords.

Table 3.9. Top 25 genes with lower mRNA expression in Holsteins compared to Herefords ordered by the amount of fold change. Fifteen of the top 25 genes with lower expression in Holsteins were found to be related to cell differentiation and proliferation, system development and growth, particular the musculoskeletal and nervous system (*TNC*, *IQGAP3*, *CDC20, EN1*, *SLITRK4*, *ACTG2*, *DES*, *MBNL3*). Four genes related to lipid metabolism were also listed (*C19orf80*, *ASIP*, *ACLY*, *INSIG1*).

Ensembl ID	Log FC	Official symbol	Gene description
ENSBTAG00000003989	5.77133	*GSTO1*	Glutathione S-transferase ω 1
ENSBTAG00000017040	4.66857	*LY6E*	Lymphocyte antigen 6 complex, locus E
ENSBTAG00000002786	4.22264	*LOC781494*	Myeloid-associated differentiation marker-like
ENSBTAG00000009570	3.98740	*C19orf80*	Chromosome 19 open reading frame 80
ENSBTAG00000034077	3.91406	*ASIP*	Agouti signaling protein
ENSBTAG00000015441	3.84480	*ACTG2*	Actin, γ 2
ENSBTAG00000000575	3.84335	*TNC*	Tenascin C
ENSBTAG00000016740	3.53704	*ACLY*	ATP citrate lyase
ENSBTAG00000015988	3.52244	*MYH11*	Myosin-11
ENSBTAG00000007237	3.50522	*BUB1B*	Mitotic checkpoint serine/threonine kinase B
ENSBTAG00000006882	3.49885	*IQGAP3*	IQ motif containing GTPase activating protein 3
ENSBTAG00000009819	3.35071	*CDC20*	Cell division cycle 20
ENSBTAG00000030483	3.33782	*KLK7*	Kallikrein-related peptidase 7
ENSBTAG00000001592	3.31603	*INSIG1*	Insulin induced gene 1
ENSBTAG00000005353	3.31146	*DES*	Desmin
ENSBTAG00000001785	3.26381	*TGM3*	Transglutaminase 3 (E polypeptide, protein-glutamine-γ-glutamyltransferase)
ENSBTAG00000025181	3.24708	*CCDC85C*	Coiled-coil domain containing 85C
ENSBTAG00000008105	3.22040	*RBM38*	RNA binding motif protein 38
ENSBTAG00000000590	3.19727	*POLE*	Polymerase (DNA directed), ϵ, catalytic subunit
ENSBTAG00000014088	3.18739	*MBNL3*	Muscleblind-like splicing regulator 3
ENSBTAG00000002444	3.15383	*MKI67*	Marker of proliferation Ki-67
ENSBTAG00000012640	3.13714	*S100A8*	S100 calcium binding protein A8
ENSBTAG00000005260	3.12298	*SPP1*	Secreted phosphoprotein 1
ENSBTAG00000021494	3.09230	*EN1*	Engrailed homeobox 1
ENSBTAG00000011780	3.04418	*SLITRK4*	SLIT and NTRK-like family, member 4

Table 3.10. Top 25 genes with higher mRNA expression in Holsteins compared to Herefords ordered by the amount of fold change. A number of the genes with higher expression in Holsteins were involved in absorption, synthesis, release, and transport of nutritional elements (*RYR3*, *GIF*, *RDH16*, *SLC16A12*, *FABP7*, *NTS*). Genes were also implicated in immune reactions (*TNFAIP6*, *IRF4*, *SLAMF9*, *IL1RL1*), cell proliferation (*HES6*, *PPP2R2B*, *NOX4*, *GRB7*), and reproductive organ functions (*OOSP1*, *AKAP4*).

Ensembl ID	Log FC	Official symbol	Gene description
ENSBTAG00000012057	-8.74203	*GIF*	Gastric intrinsic factor (vitamin B synthesis)
ENSBTAG00000019870	-7.03131	*SLC14A1*	Solute carrier family 14 (urea transporter), member 1
ENSBTAG00000005305	-5.37334	*NTS*	Neurotensin
ENSBTAG00000027348	-5.35623	*OOSP1*	Oocyte-secreted protein 1
ENSBTAG00000007239	-5.25893	*TNFAIP6*	Tumour necrosis factor, α-induced protein 6
ENSBTAG00000002929	-4.62352	*IRF4*	Interferon regulatory factor 4
ENSBTAG00000016399	-4.43370	*AKAP4*	A kinase (PRKA) anchor protein 4
ENSBTAG00000031532	-4.06503	*DACT2*	Dishevelled-binding antagonist of β-catenin 2
ENSBTAG00000001392	-4.03295	*RDH16*	Retinol dehydrogenase 16 (all-trans)
ENSBTAG00000046420	-3.96084	*SNORD113*	Small nucleolar RNA SNORD113/SNORD114 family
ENSBTAG00000009870	-3.80481	*HES6*	Hes family bHLH transcription factor 6
ENSBTAG00000015374	-3.51475	*COL22A1*	Collagen, type XXII, α 1
ENSBTAG00000018540	-3.43827	*NOX4*	NADPH oxidase 4
ENSBTAG00000007241	-3.36955	*SLAMF9*	SLAM family member 9
ENSBTAG00000001862	-3.21030	*PPP2R2B*	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform
ENSBTAG00000017366	-3.18885	*GRB7*	Growth factor receptor-bound protein 7
ENSBTAG00000033803	-3.17693	*FABP7*	Fatty acid binding protein 7
ENSBTAG00000012071	-3.10848	*ASGR1*	Asialoglycoprotein receptor 1
ENSBTAG00000004662	-3.09386	*SLC16A12*	Solute carrier family 16, member 12
ENSBTAG00000043378	-2.98098	*SNORD81*	Small nucleolar RNA
ENSBTAG00000004355	-2.93394	*ROBO3*	Roundabout, axon guidance receptor, homolog 3
ENSBTAG00000018571	-2.93354	*IL1RL1*	Interleukin 1 receptor-like 1
ENSBTAG00000011002	-2.92651	*CCDC136*	Coiled-coil domain containing 136
ENSBTAG00000002215	-2.89505	*GFPT2*	Glutamine-fructose-6-phosphate transaminase 2
ENSBTAG00000025642	-2.86489	*RYR3*	Ryanodine receptor 3

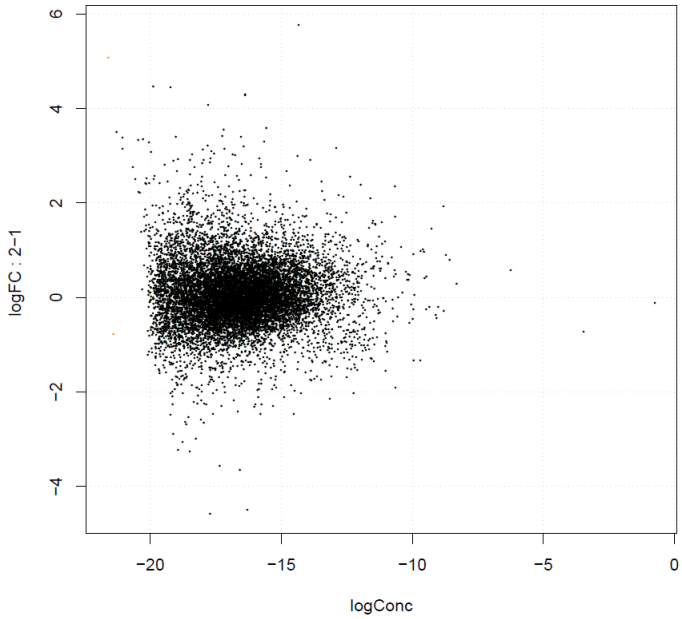


Figure 3.3. Smear plot comparing log FC and –log average gene expression counts on DEG of AT in Holsteins versus Herefords. This plot shows distribution of annotated genes.

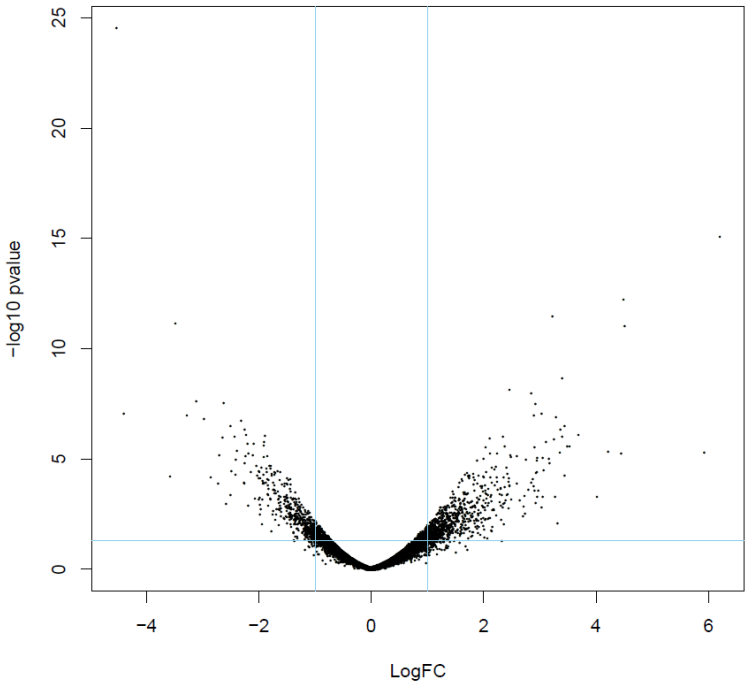


Figure 3.4. Volcano plot comparing -log10 p-value and log FC on DEG of AT in Holsteins versus Herefords. Volcano plot with DE genes of interest with FC $\geq$ 2 in the upper left and right regions.

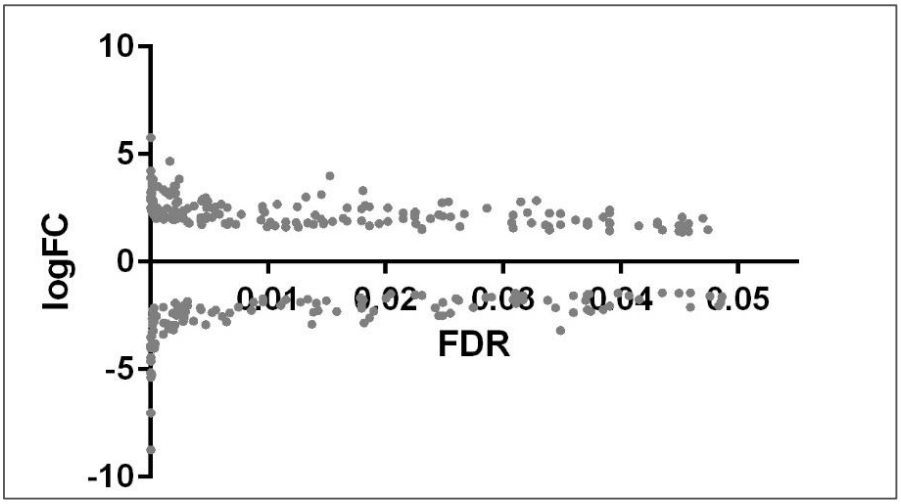


Figure 3.5. Differential gene distribution by log FC versus FDR on DEG of AT in Holsteins versus Herefords.

3.3.1.1.3 Hierarchical cluster analysis and gene ontology annotation

Hierarchical clustering showed an overview of global gene expression of peri-renal AT of Holsteins versus Herefords (Figure 3.6). The heat map shows genes differentially expressed between breeds, where red represents genes with higher expression level, and green the genes with lower expression. Of 24,616 genes in the annotated genome, 2,853 satisfied the initial QC criteria of read counts $\geq$ 50, and FC $\geq$ 2.0. Results showed that expression of genes from the replicates was specifically clustered into the respective breeds as anticipated. Clustering analysis was run on transcription levels of peri-renal AT from several beef and dairy breeds, and results showed that beef and dairy breeds were distinctively segregated (Appendix 6). However as only single samples were available for Charolais, Limousin, Highland and Jersey cattle, these results served as a preliminary analysis.

The gene ontology (GO) analysis was performed using Directed Acyclic Graphs (DAGs) produced by WebGestalt (Figure 3.7). The human database was used as reference in this analysis as cattle were not among the species supported by the database depository. In order to accomplish the analysis, the cattle Ensembl identifiers were matched to their respective human orthologues where a 1:1 orthologue exists. During the process, 51 genes were lost from the original 311 where appropriate matches could not be made. The resulting DAGs showed that enriched GO terms of differentially expressed genes (DEG) in Holsteins compared to Herefords were categorised under the biological process and cellular component sections. The GO terms shown in red were significantly enriched terms (Figure 3.7). Terms under Biological Process are

multicellular organismal process (GO:0032501), single-multicellular organism process (GO:0044707), while only one was significant under the Cellular Component: extracellular region (GO:0005576).

The GO terms definition according to European Molecular Biology Laboratory, The European Bioinformatics Institute (EMBL-EBI); (http://www.ebi.ac.uk/QuickGO/GTerm) database on multicellular organismal process refers to biological processes that occur at organism level, particularly on regulatory functions in organism reproduction. Single-multicellular organism process is related to post embryonic development and growth of multiple organs in general, including circadian regulation of gene expression and physiological functions of body systems. Extracellular region refers to processes occurring out of cell plasma membrane, as well as in the extracellular organelles. Carbohydrate and amino acid transport, regulation of signal transduction are among the processes cited to occur in the extracellular region.

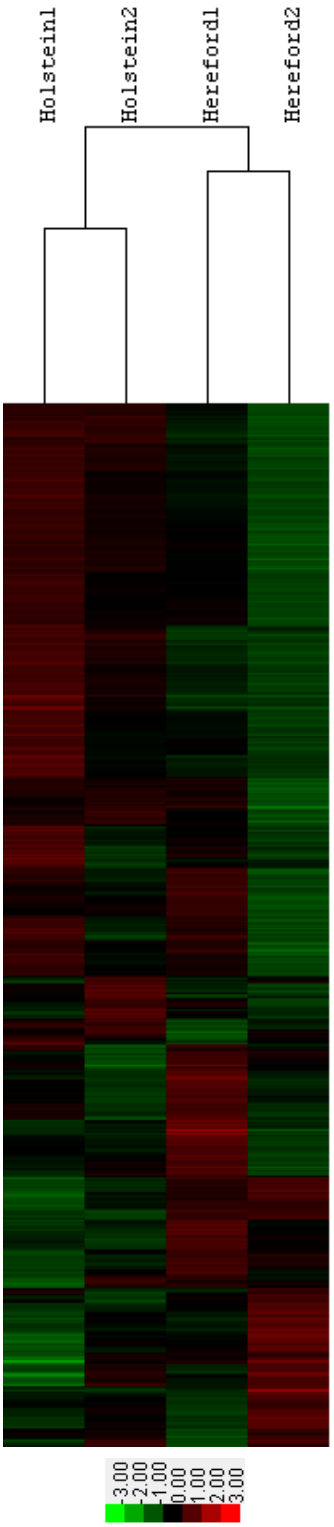


Figure 3.6. Hierarchical clustering of global gene expression detected through RNA-seq in peri-renal AT of Holsteins versus Herefords. Heat map shows genes differentially expressed between breeds, red represent upregulated genes, and green the downregulated genes. 2,853 genes from the total of 24,616, satisfied the initial QC criteria of all samples with read counts $\geq$ 50, and FC $\geq$ 2. Results showed that gene expressions from the replicates were specifically clustered in their respective breed. Holstein n = 2, Hereford n = 2.

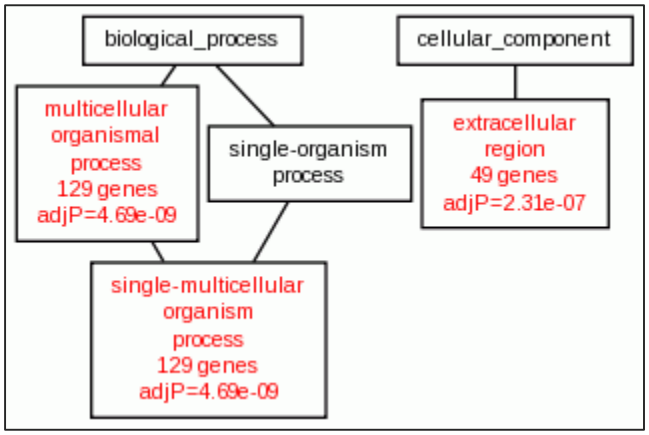


Figure 3.7. WebGestalt Directed Acyclic Graphs (DAGs) showing enriched GO categories under Biological Process, and Cellular Component of differentially expressed genes for Holsteins compared to Herefords by fold change. GO term enrichments are shown in red. Enriched GO terms included biological processes occurring within both cellular and extracellular levels, involving development and growth of multiple organs in general.

3.3.1.1.4 Pathway analysis

Pathway analysis was conducted using Ingenuity® Pathway Analysis (http://www.ingenuity.com/products/ipa). The diagram highlights interaction networks of genes of interest in AT of Holstein and Hereford cattle (Figure 3.8). Other genes of importance and nutritional pathways for the fat soluble vitamins calcitriol (vitamin D) and all-*trans* retinoic acid (ATRA, vitamin A) were incorporated. The pathway diagram was overlaid with DEG list comparing Holsteins and Herefords, where the higher (red) or lower (green) levels of gene expression referred to that of the Herefords. Icons in red and green represented higher and lower expressed genes found in the DEG list respectively, while the ones in white, orange and blue were additional genes associated with functions related to pathway of interest. Intensity of these colours also correlated to the degree of DE between the breeds. Genes or molecules coloured in orange were predicted to activate downstream targeted molecules, and the ones in blue were predicted to elicit inhibitory reactions. Solid lines connecting the genes and molecules indicated direct interactions, whereas broken lines indicated presence of intermediate genes or molecules, but these were not shown in the network diagram. Likewise, orange lines indicated an activation relationship, and lines in blue were inhibitory, while the ones in yellow indicated inconsistent relationship to the state of downstream gene or molecule with reference to Ingenuity® Knowledge Base. Shape of the end of lines also depicted the predicted relationship between genes and molecules, arrow symbolise activation whilst a short perpendicular line indicated inhibition.

The pathway diagram directly reflects the interactions of DEG identified through RNA-seq between Hereford and Holstein cattle (Figure 3.8). Genes with higher mRNA expression in Herefords were *LEP*, *FASN*, *SCD*, *ACACA*, *INSIG1*, *ELOVL5*, *ACLY*, *RAN*, *RXRA*, *SREBP1* (*SREBF1*), and *SREBP2* (*SREBF2*). By contrast, *LEPR* and *IRF4* mRNA had higher expression in the Holsteins. The genes listed above are genes reported to be involved in enhancing or suppressing adipogenesis and lipogenesis, which will be discussed in further detail in later sections. However genes that are actively involved in adipocyte proliferation and differentiation such as *PPARγ*, *CEBPα*, *MAPK*, and *STAT3* (Rosen and MacDougald, 2006, Kim et al., 2012, Przybyt et al., 2013), were not differentially expressed between the breeds.

Higher *LEP* expression was observed in Hereford compared to Holstein cattle, although expression of leptin receptor (*LEPR*) expression appeared to be lower. As *LEP* acts via its receptor, the effects of *LEP* are likely to be depressed locally (Chelikani et al., 2003), however it may still exert its effect through *LEPR* found at the hypothalamus (Leinninger et al., 2009). Both β actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*G3PD* or *GAPDH*) which are commonly used reference genes in qRT-PCR, were observed to be differentially expressed and were interacting with other genes in the network. Thus, these were unsuitable to be used as reference genes in this study.

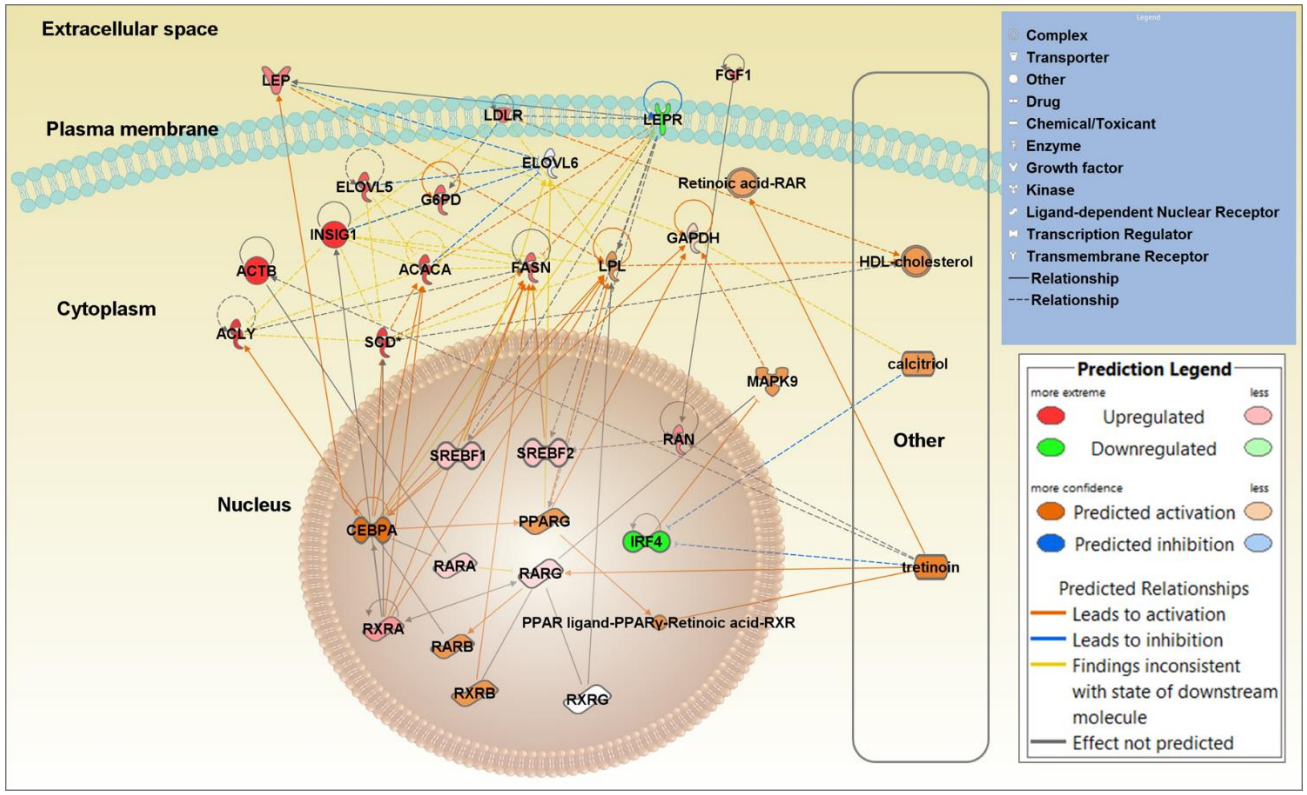


Figure 3.8. Ingenuity® Pathway Analysis highlighting interacting networks of genes of interest between Holstein and Hereford. The diagram was overlaid with DEG between Holstein and Hereford AT, where levels of gene expression refers to that of Herefords. Icons in red and green represented higher and lower expressions of genes found in the DEG list respectively, while the ones in orange and blue were genes not included in the list, but associated with functions related to pathway of interest. Genes and lines coloured in orange and blue were predicted to activate or inhibit downstream targeted molecules correspondingly, while yellow lines indicated inconsistent relationship to the state of downstream gene with reference to Ingenuity® Knowledge Base. An arrow shape of the end of a line depicted activation whilst a short perpendicular line indicated inhibition action of the gene.

3.3.1.1.5 Quantitative reverse transcription PCR validation

Several genes of interest were selected for validation with qRT-PCR (Table 3.11). Genes were selected based on their reported functions or influence on nutrition, fat metabolism, energy expenditure, adipogenesis and lipogenesis. Based on the RNA-seq data, mRNA expression of *ELOVL5*, *INSIG1*, *LDLR*, *RAN*, *RXRα*, and *SCD* were higher in Herefords as compared to Holsteins, whereas *IRF4* and *LEPR* were lower (Table 3.11). The qRT-PCR was performed using cDNA of 6 biologically independent samples from each breed. The TATA box binding protein (*TBP*) and ubiquitously-expressed, prefoldin-like chaperone (*UXT*) were used as reference genes. These reference genes were selected from the literature (Taga et al., 2012a, Bonnet et al., 2013), and expression was compared across all samples to ensure their stability as reference gene. The cycle threshold (Ct) values of genes of interest were normalised against *TBP* and *UXT*, and the mean from both were taken as the expression value for the particular gene in each replicates. Results obtained from qRT-PCR confirmed that expression of the genes of interest was consistent with the results obtained by RNA-seq (Table 3.11).

Table 3.11. Gene expression FC between breeds through RNA-seq. *ELOVL5*, *INSIG1*, *LDLR*, *RAN*, *RXRα*, and *SCD* expression was lower in Holstein compared to Hereford, while expression of *IRF4* and *LEPR* was higher.

Genes	Hereford vs Holstein Log FC (FDR < 0.05)
ELOVL fatty acid elongase 5 *(ELOVL5*)	2.54
Insulin induced gene 1 (*INSIG1*)	3.31
Interferon regulatory factor 4 (*IRF4*)	-4.62
Low density lipoprotein receptor (*LDLR*)	2.05
Leptin receptor (*LEPR*)	-2.50
RAN, member RAS oncogene family (*RAN*)	1.81
Retinoid X receptor, alpha (*RXRα*)	1.56
Stearoyl-CoA desaturase (*SCD*)	3.00

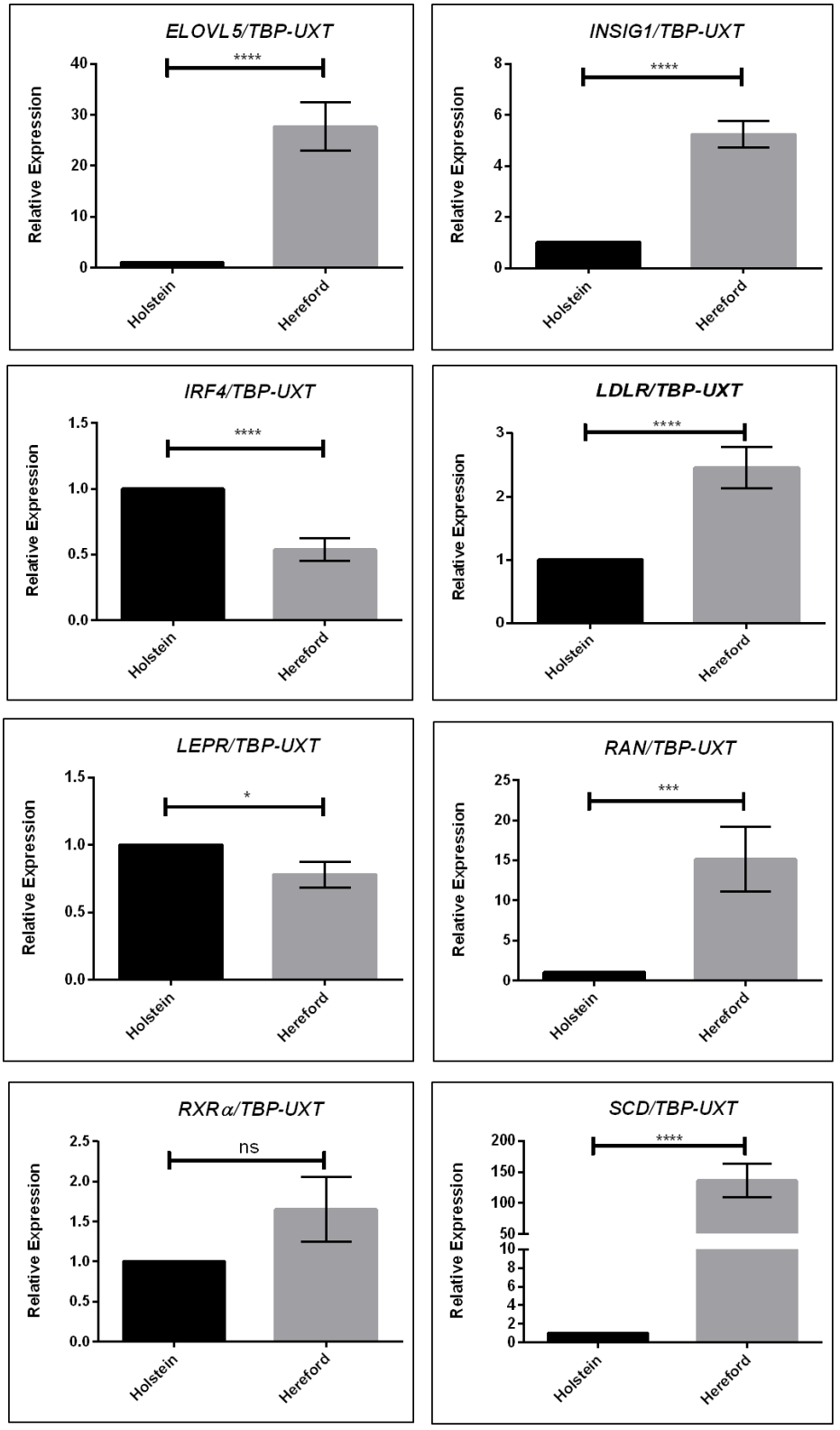


Figure 3.9. qRT-PCR validation of mRNA expression from peri-renal AT between Holstein and Hereford. These results were consistent with RNA-seq for genes of interest in the study except for *RXRα*. Mean values ± SEM. Level of significance: ns = non-significant, * = $p < 0.05$, *** = $p < 0.0005$, **** = $p < 0.0001$. Holstein n = 6, Hereford n = 6.

3.3.1.2 LP versus HP

3.3.1.2.1 Statistics and read annotations

As before, paired sequence reads were produced using Illumina Hi-Seq platform for RNA-seq analysis. The number of raw reads and numbers of reads retained following quality control and mapping processes are shown in Table 3.12 and Figure 3.10. The percentages of aligned and QC reads were similar in all samples (Figure 3.11). A total of 11,902 of genes were annotated to *Bos taurus* genome in Ensembl database from the mapped reads, and these were used for subsequent analyses. An overall high percentage of reads were successfully mapped to the reference genome, averaging around 94.3%, and the highest percentage of reads not usable for analysis was at 6.0% (LP2); (Table 3.12).

Table 3.12. Breakdown of RNA-seq reads for adipose samples of LP and HP. Number of raw reads generated from RNA-seq are shown as total reads. QC reads are sequence reads kept after QC, and the ones that did not pass the QC processes are the discarded reads. Sequence reads that were confidently mapped to reference genome are presented as mapped reads, leaving the ones that were not mapped as unmapped reads.

ID	Breed	Age	Total reads	QC reads	Mapped reads	Unmapped reads	Discarded reads
LP1	AAX	2y 5m	62,147,636 (100%)	60,968,340 (98.10%)	58,547,849 (94.21%)	2,420,491 (3.89%)	1,179,296 (1.90%)
LP2	AAX	2y 1m	53,098,498 (100%)	52,239,154 (98.38%)	49,910,916 (94.00%)	2,328,238 (4.38%)	859,344 (1.62%)
HP1	AAX	2y 0m	59,834,320 (100%)	58,914,592 (98.46%)	56,534,520 (94.49%)	2,380,072 (3.98%)	919,728 (1.54%)
HP2	AAX	1y 10m	60,201,478 (100%)	59,151,582 (98.25%)	56,991,289 (94.67%)	2,160,293 (3.59%)	1,049,896 (1.74%)

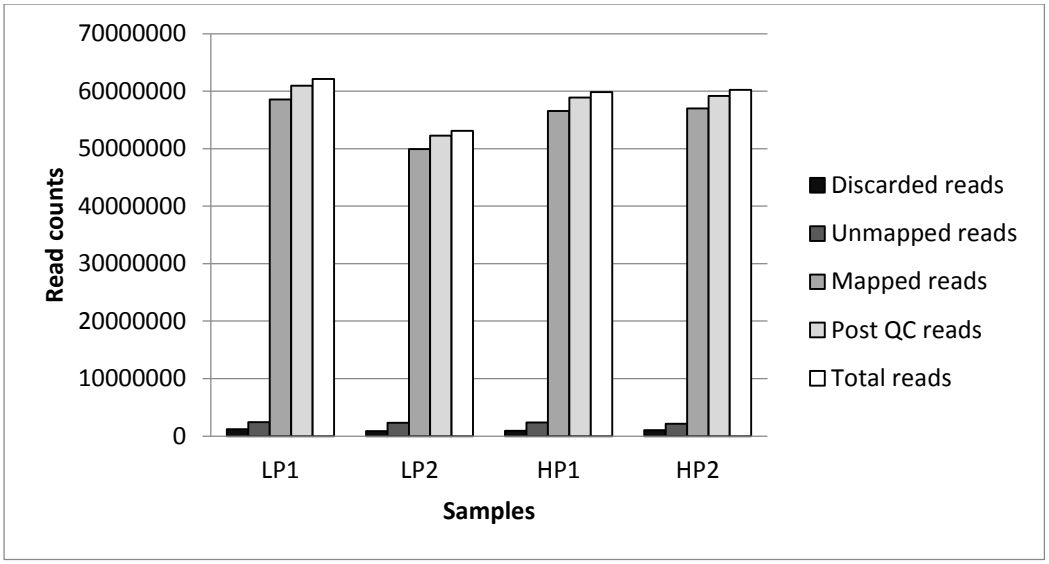


Figure 3.10. RNA-seq reads dispersions by count numbers for adipose samples of LP and HP. Total reads refer to the number of raw reads generated from RNA-seq. Post QC reads are sequence reads that passed QC criteria, and the discarded reads were the ones that failed to pass QC. Mapped reads are sequence reads confidently mapped to reference genome, while the ones that were not mapped are presented as unmapped reads. All samples were sequenced once, although LP1 and LP2 appeared to have a slightly higher or lower number of counts respectively.

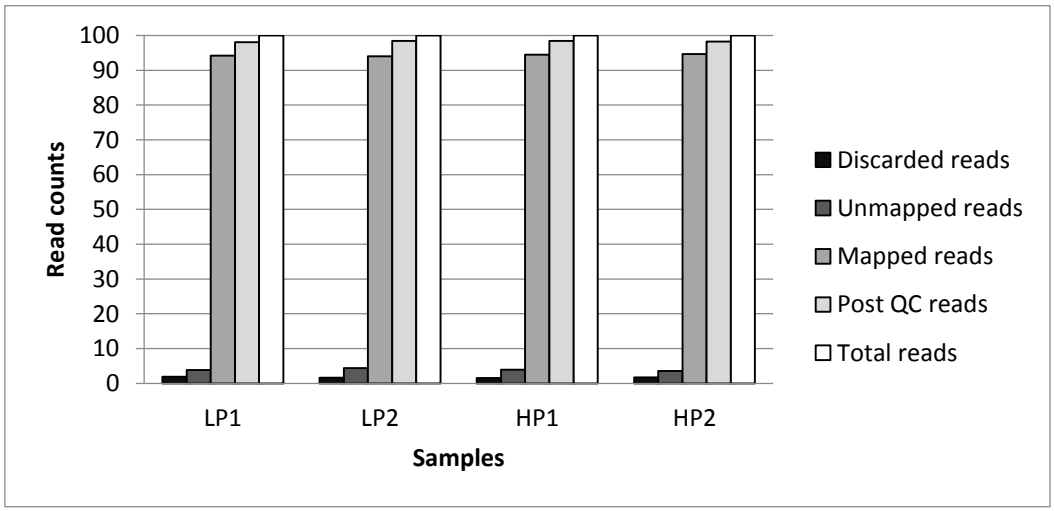


Figure 3.11. RNA-seq reads dispersions by percentages of count numbers for adipose samples of LP and HP. This graph shows the breakdown of sequence reads in percentages corresponding to count numbers presented in Figure 3.10. Compositions of LP2 appeared comparable with other samples although the count number was lower.

3.3.1.2.2 Differentially expressed genes

Expression of a total of 176 genes was found to be significantly different between peri-renal AT of LP and HP through DE analysis using edgeR (FC $\geq$ 2, FDR < 0.05). Among these, 74 were highly expressed in HP, while 102 genes showed higher expression in LP (Appendix 3). Genes were prioritized for subsequent analysis and validation by having FC $\geq$ 2 and FDR < 0.05 (Figures 3.12, 3.13 and 3.14).

Cell proliferation and growth are the dominant functions cited for genes listed on the top 25 genes with lower expression in LP compared to HP: *PIK3C2A*, *RASD1*, *P2RY2*, *PHLDA1* (Ng et al., 2009, Vaidyanathan et al., 2004, Xie et al., 2014, Battistella et al., 2014), according to information obtained from NCBI Reference Sequence Database, RefSeq (http://www.ncbi.nlm.nih.gov/refseq/); (Table 3.13). Some of the genes listed are involved in development of muscle: *ZBED6* and *PRG4* (Markljung et al., 2009, Samsom et al., 2014) and reproductive organs: *SPATA16* and *SLC6A6* (Dam et al., 2007, Desforges et al., 2013). Genes implicated in lipid metabolism were also among the top 25 listed (*LEPR*, *ABCD2*, *GPR41* (*FFAR3*), *PER2*, *GIPR*).

Genes listed on the list of top 25 genes with higher expression in LP versus HP were indicated to be involved in physiological functions across various tissues, including cell proliferation, carcinogenic markers, immune response, reproduction, muscle formation and neuronal excitability (Table 3.14). A number of genes were particularly involved in AT development: *SCD*, *FASN*, *UCP1*, *HSD17B14*, and *C19orf80* (Kim and Spiegelman, 1996, Kersten, 2001,

Kozak and Anunciado-Koza, 2008, Duijvesteijn et al., 2010, Strable and Ntambi, 2010, Li and Cheng, 2014, Barja-Fernandez et al., 2015).

Table 3.13. Top 25 genes with lower mRNA expression in LP compared to HP ordered by the amount of fold change. Cell proliferation and growth was the dominant functions indicated for genes in this list (*PIK3C2A*, *RASD1*, *P2RY2*, and *PHLDA1)*, a few particularly involving muscles (*ZBED6* and *PRG4*) and reproductive organs (*SPATA16* and *SLC6A6*). Genes implicated in lipid metabolism were among the top 25 listed (*LEPR*, *ABCD2*, *GPR41*, *PER2*, *GIPR*).

Ensembl ID	Log FC	Official symbol	Gene description
ENSBTAG00000003212	3.71574	*NNAT*	Neuronatin
ENSBTAG00000005910	3.46382	*LEPR*	Leptin receptor
ENSBTAG00000007846	2.62749	*ITIH3*	Inter-α-trypsin inhibitor heavy chain 3
ENSBTAG00000012715	2.59606	*KIF26B*	Kinesin family member 26B
ENSBTAG00000017369	2.39055	*MAMDC2*	MAM domain containing 2
ENSBTAG00000016768	1.93776	*SCN3B*	Sodium channel, voltage-gated, type III, β
ENSBTAG00000045702	1.91936	*ZBED6*	Zinc finger, BED-type containing 6
ENSBTAG00000015214	1.84383	*CA3*	Carbonic anhydrase III,
ENSBTAG00000031802	1.81072	*SPATA16*	Spermatogenesis associated 16
ENSBTAG00000011088	1.73046	*SLC6A6*	Solute carrier family 6 (neurotransmitter transporter), member 6
ENSBTAG00000038043	1.68972	*ABCD2*	ATP-binding cassette, sub-family D (ALD), member 2
ENSBTAG00000004901	1.66074	*PIK3C2A*	Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 alpha
ENSBTAG00000001879	1.64991	*PER2*	Period homolog 2
ENSBTAG00000044208	1.64518	*DUSP4*	Dual specificity phosphatase 4
ENSBTAG00000002996	1.64225	*SHROOM4*	Shroom family member 4
ENSBTAG00000011932	1.63503	*PRG4*	Proteoglycan 4 precursor
ENSBTAG00000005527	1.61867	*GIPR*	Gastric inhibitory polypeptide receptor
ENSBTAG00000020520	1.59728	*RASD1*	RAS, dexamethasone-induced 1
ENSBTAG00000039050	1.57189	*P2RY2*	Purinergic receptor P2Y, G-protein coupled, 2
ENSBTAG00000007843	1.55726	*ITIH1*	Inter-α-trypsin inhibitor heavy chain 1
ENSBTAG00000013791	1.53872	*GPR41*	Free fatty acid receptor 3
ENSBTAG00000010018	1.51965	*ATP7A*	ATPase, Cu^{2+} transporting, alpha polypeptide
ENSBTAG00000034985	1.51190	*PHLDA1*	Pleckstrin homology-like domain, family A, member 1
ENSBTAG00000009691	1.45732	*SH2B2*	SH2B adaptor protein 2
ENSBTAG00000033429	1.42279	*FAM229B*	Protein FAM229B

Table 3.14. Top 25 genes with higher mRNA expression in LP compared to HP ordered by the amount of fold change. Genes listed here have been indicated to be involved in physiological functions across various tissues, including cell proliferation, carcinogenic markers, immune response, reproduction, muscle formation and neuronal excitability. A number of genes were also involved in AT development (*SCD*, *FASN*, *UCP1*, *HSD17B14*, and *C19orf80*).

Ensembl ID	Log FC	Official symbol	Gene description
ENSBTAG00000021310	-4.21481	*COL4A4*	Collagen, type IV, α 4
ENSBTAG00000038584	-4.16615	*OLFM1*	Olfactomedin 1
ENSBTAG00000034498	-3.44515	*LY6D*	Lymphocyte antigen 6 complex, locus D
ENSBTAG00000004647	-3.01268	*UCP1*	Uncoupling protein 1
ENSBTAG00000036343	-2.82371	*LOC526488*	Heat shock transcription factor, Y-linked-like protein-like
ENSBTAG00000000828	-2.74837	*CAPN6*	Calpain 6
ENSBTAG00000017007	-2.73271	*TRIB3*	Tribbles homolog 3
ENSBTAG00000012991	-2.47261	*PRUNE2*	PRUNE2 protein
ENSBTAG00000009570	-2.43596	*C19orf80*	Chromosome 19 open reading frame 80
ENSBTAG00000015080	-2.32235	*PHACTR3*	Phosphatase and actin regulator 3
ENSBTAG00000002075	-2.29908	*MME*	Membrane metallo-endopeptidase
ENSBTAG00000030322	-2.27532	*KLHDC7A*	Kelch domain containing 7A
ENSBTAG00000009174	-2.23870	*HSD17B14*	Hydroxysteroid (17-beta) dehydrogenase 14
ENSBTAG00000009831	-2.20338	*OCA2*	Oculocutaneous albinism II
ENSBTAG00000038058	-2.15803	*LOC518623*	Glycine-N-acyltransferase-like
ENSBTAG00000001021	-2.15480	*CYP1A1*	Cytochrome P450
ENSBTAG00000011076	-2.10773	*KCNN2*	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2
ENSBTAG00000000284	-2.05034	*NAALAD2*	N-acetylated alpha-linked acidic dipeptidase 2
ENSBTAG00000045728	-2.03470	*SCD*	Stearoyl-CoA desaturase
ENSBTAG00000047957	-1.99466	*SCD*	Stearoyl-CoA desaturase
ENSBTAG00000015980	-1.98932	*FASN*	Fatty acid synthase
ENSBTAG00000021033	-1.90196	*SGK2*	Serum/glucocorticoid regulated kinase 2
ENSBTAG00000006991	-1.83545	*ADH6*	Alcohol dehydrogenase 6 (class V)
ENSBTAG00000006349	-1.80828	*EXTL1*	Exostoses (multiple)-like 1
ENSBTAG00000004510	-1.78262	*SARDH*	Sarcosine dehydrogenase

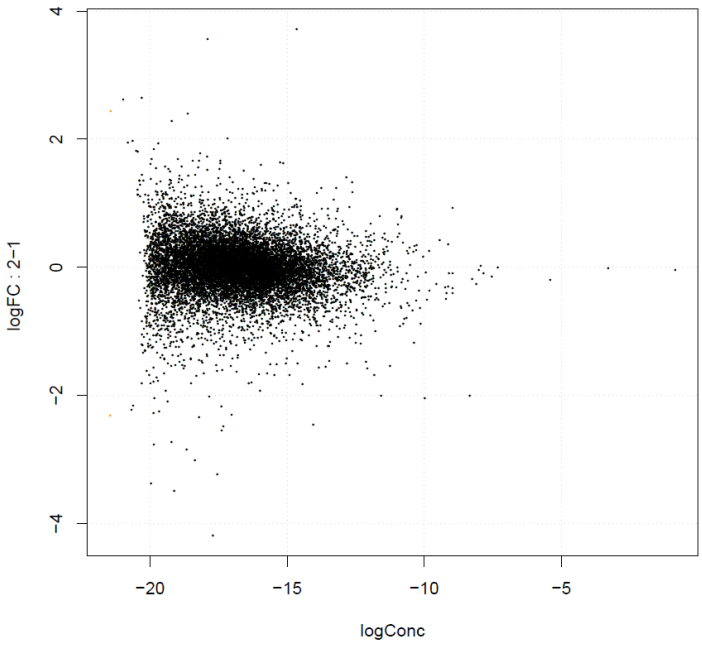


Figure 3.12. Smear plot comparing log FC and –log average gene expression counts on differential gene expression of AT between LP and HP. This plot shows distribution of annotated genes.

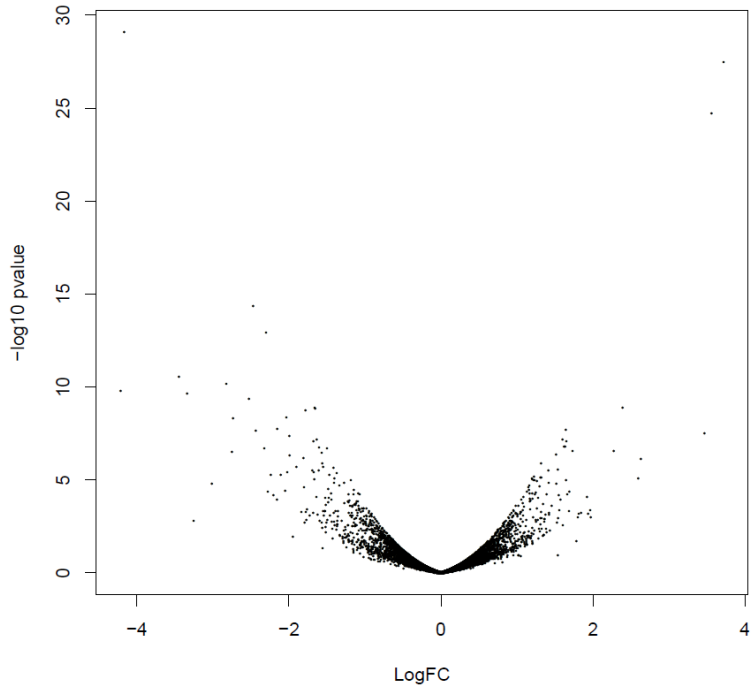


Figure 3.13. Volcano plot comparing -log10 p-value and log FC on differential gene expression of AT in LP and HP. Volcano plot with DE genes of interest with FC > 2 in the upper left and right regions.

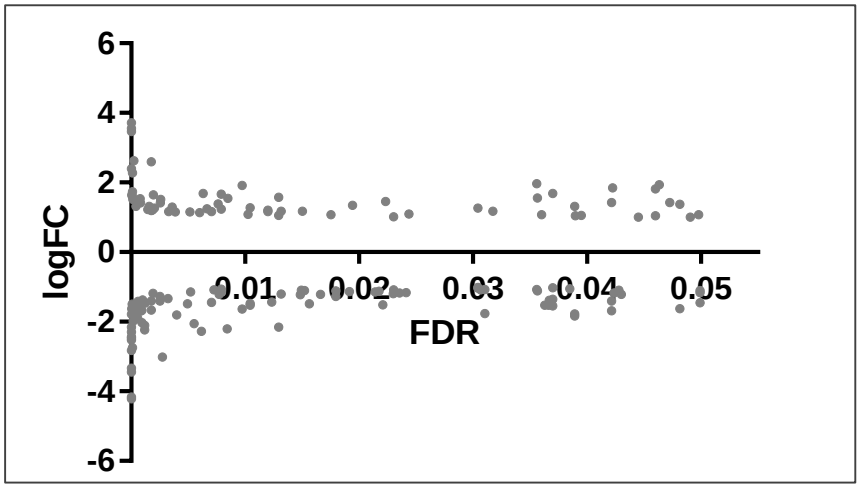


Figure 3.14. Differential gene distribution by log FC versus FDR on differential gene expression of AT in LP and HP.

3.3.1.2.3 Hierarchical cluster analysis and gene ontology anontation

Hierarchical clustering shows a global view of gene expression detected in peri-renal AT of AAX on low protein (LP) versus high protein (HP) diet through RNA-seq (Figure 3.15). As described before, the heat map shows genes differentially expressed between breeds, with red representing genes with higher expression, and green represents the genes with lower expression. A total of 813 genes, from the 24,616 annotated genes, satisfied the initial QC criteria of all samples with read counts $\geq$ 50, and FC $\geq$ 2. Results showed that mRNA expressions from animals on low and high protein diet were clustered into each respective treatment group.

The DAGs (Figure 3.16) displayed GO terms found overrepresented in the set of DEG were related to biological processes, particular in relation to fatty acid metabolism: acyl-CoA metabolic process (GO:0006637) and carboxylic acid metabolic process (GO:0019752), both derived from the 'metabolic process' node, although the former had overlapped nodes from the 'cellular process' node. Concomitantly, elements that catalysed the reactions involved in 'metabolic process' were also enriched: cofactor metabolic process (GO:0051186) that led to coenzyme metabolic process (GO:0006732), and cofactor biosynthetic process (GO:0051188), consequently coenzyme biosynthetic process (GO:0009108). These generated cofactors and coenzymes are required to catalyse processes stimulated by enzymes (from EMBL-EBI (http://www.ebi.ac.uk/QuickGO/GTerm)). In terms of small molecule metabolic process (GO:0044281) and small molecule biosynthetic process (GO:0044283), small molecules referred to molecules of low

molecular weight, monomeric and non-encoded molecule, including monosaccharide.

3.3.1.2.4 Pathway analysis

Pathway analysis was conducted using Ingenuity® Pathway Analysis (http://www.ingenuity.com/products/ipa). Interaction networks of genes of interest in AT of LP versus HP were highlighted in the diagram (Figure 3.17). A number of genes related to ATRA signalling were incorporated given their important role in development and metabolism of AT. The pathway diagram was overlaid with DEG list comparing LP and HP, where higher and lower expression of genes refers to that of HP. Colour and shape of icons and connecting lines were described in detail in previous section comparing expression levels between Holstein and Hereford cattle.

Figure 3.17 shows interactions of genes of interest between LP and HP from the list of DEG generated from RNA-seq results. Genes with higher expression in HP were *LEPR*, *ASS1*, *ID1*, *SREBP1* (*SREBF1*), *PER2*, *NNAT*, and *CA3*. *LEP* and *SREBP2* also had a higher mRNA expression in HP compared to LP, however the FC values were relatively low, as illustrated by the weak pink background of their respective icons. Meanwhile, *FASN*, *SCD*, *ACACA*, *ELOVL5*, *RXRA*, *UCP1*, *IGF2R*, *IGFBP5* and *CA2* had higher expression in LP compared to HP.

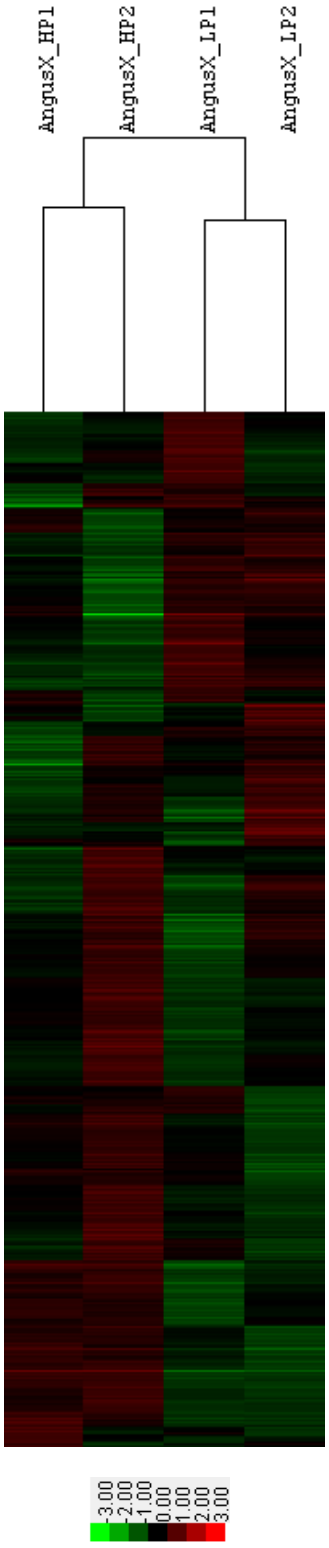


Figure 3.15. Hierarchical clustering of global gene expression detected through RNA-seq in peri-renal AT of LP and HP. Heat map shows genes differentially expressed between AT of LP and HP, where red represents genes with higher expression, and green the genes with lower levels of expression. 813 genes from the total of 24,616, satisfied the initial QC criteria of all samples with read counts $\geq$ 50, and FC $\geq$ 2. Results showed that gene expressions from the AT of LP and HP were specifically clustered into each respective treatment group. LP n = 2, HP n = 2.

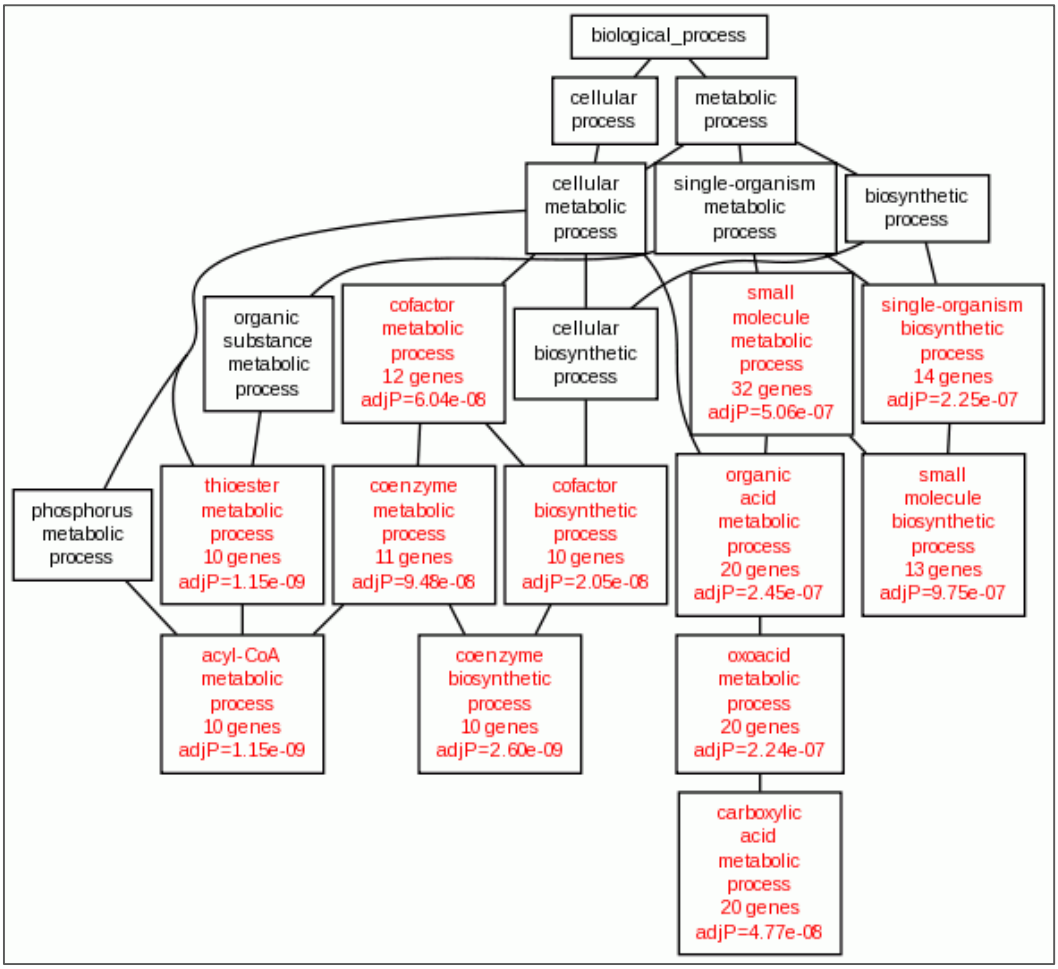


Figure 3.16. WebGestalt Directed Acyclic Graphs (DAGs) showing enriched GO categories under Biological Process of genes with lower mRNA expression for LP compared to HP by FC. GO term enrichments are shown in red. Enriched terms appeared to be related to fatty acid metabolism and production of cofactors and coenzymes that are critical in aid of promoting catalytic processes induced by enzymes.

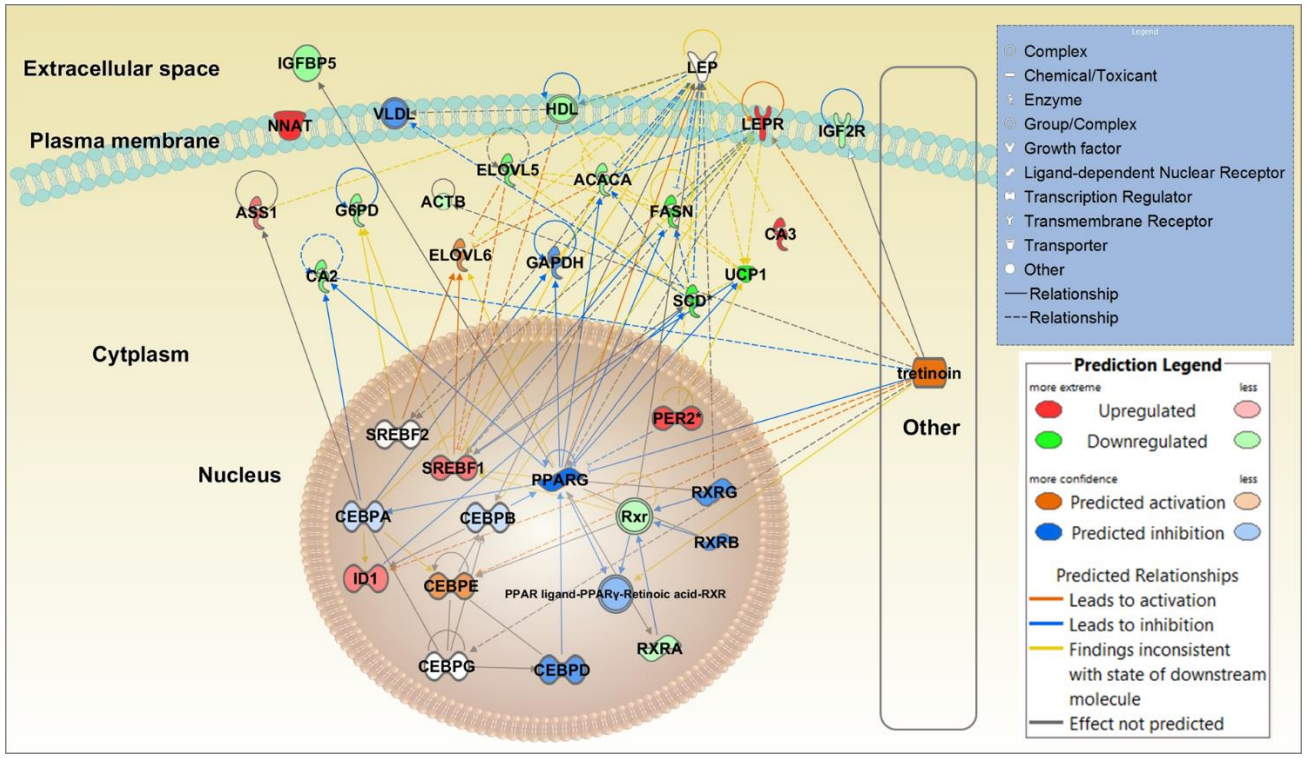


Figure 3.17. Ingenuity® Pathway Analysis highlighting interacting networks of genes of interest between LP and HP. This diagram was overlaid with DEG between LP and HP, where the higher and lower gene expressions refers to that of HP. Icons in red and green represents genes with higher and lower expression in the DEG list respectively, while the ones in orange and blue were genes not included in the list, but associated with functions related to pathway of interest. Genes and lines coloured in orange and blue were predicted to activate or inhibit downstream targeted molecules correspondingly, while lines in yellow indicated inconsistent relationship to the state of downstream gene with reference to Ingenuity® Knowledge Base. Arrow shape of the end of lines depicted activation relationship between genes, whilst a short perpendicular line indicated inhibition.

3.3.1.2.5 Quantitative reverse transcription PCR validation

Genes of interest selected for validation with qRT-PCR are listed in Table 3.15. These genes were chosen based on their reported functions or influence on fat metabolism, energy expenditure, differentiation and development of adipocytes, also in relation to protein turnover and amino acid catabolism. Values of log FC were also taken into consideration, as it may be likely that higher FC will lead to more evident differences in biological functions, although this is not always guaranteed.

Higher mRNA expression of *ASS1*, *LEPR*, *NNAT* and *PER2* was detected in HP, whereas expression of the *CA2*, *ELOVL5*, *ELOVL6*, *FASN* and *IGFBP5* genes was higher in LP (Table 3.15). Validation of RNA-seq results was conducted utilising qRT-PCR. The cDNA of 6 replicates from each dietary treatment group were used, with *TBP* and *UXT* as the reference genes. Results from qRT-PCR were able to validate transcriptomic expression of *ASS1*, *CA2*, *LEPR*, *NNAT*, and *PER2*, however *ELOVL5*, *ELOVL6*, *FASN*, and *IGFBP5* were not found to be differentially expressed (Figure 3.18).

Table 3.15. Gene expression FC between LP and HP through RNA-seq. *ASS1*, *LEPR*, *NNAT* and *PER2* expression was lower in LP compared to HP, while expression of *CA2*, *ELOVL5*, *ELOVL6*, *FASN* and *IGFBP5* was higher.

Genes	HP vs LP Log FC (FDR < 0.05)
Argininosuccinate synthase 1 *(ASS1*)	1.30
Carbonic anhydrase (*CA2*)	-1.41
ELOVL fatty acid elongase 5 *(ELOVL5*)	-1.48
ELOVL fatty acid elongase 6 *(ELOVL6*)	-1.46
Fatty acid synthase (*FASN*)	-1.98
Insulin-like growth factor binding protein 5 (*IGFBP5*)	-1.02
Leptin receptor (*LEPR*)	3.46
Neuronatin (*NNAT*)	3.71
Period homolog 2 (*PER2*)	1.64
Stearoyl-CoA desaturase (*SCD*)	-1.99

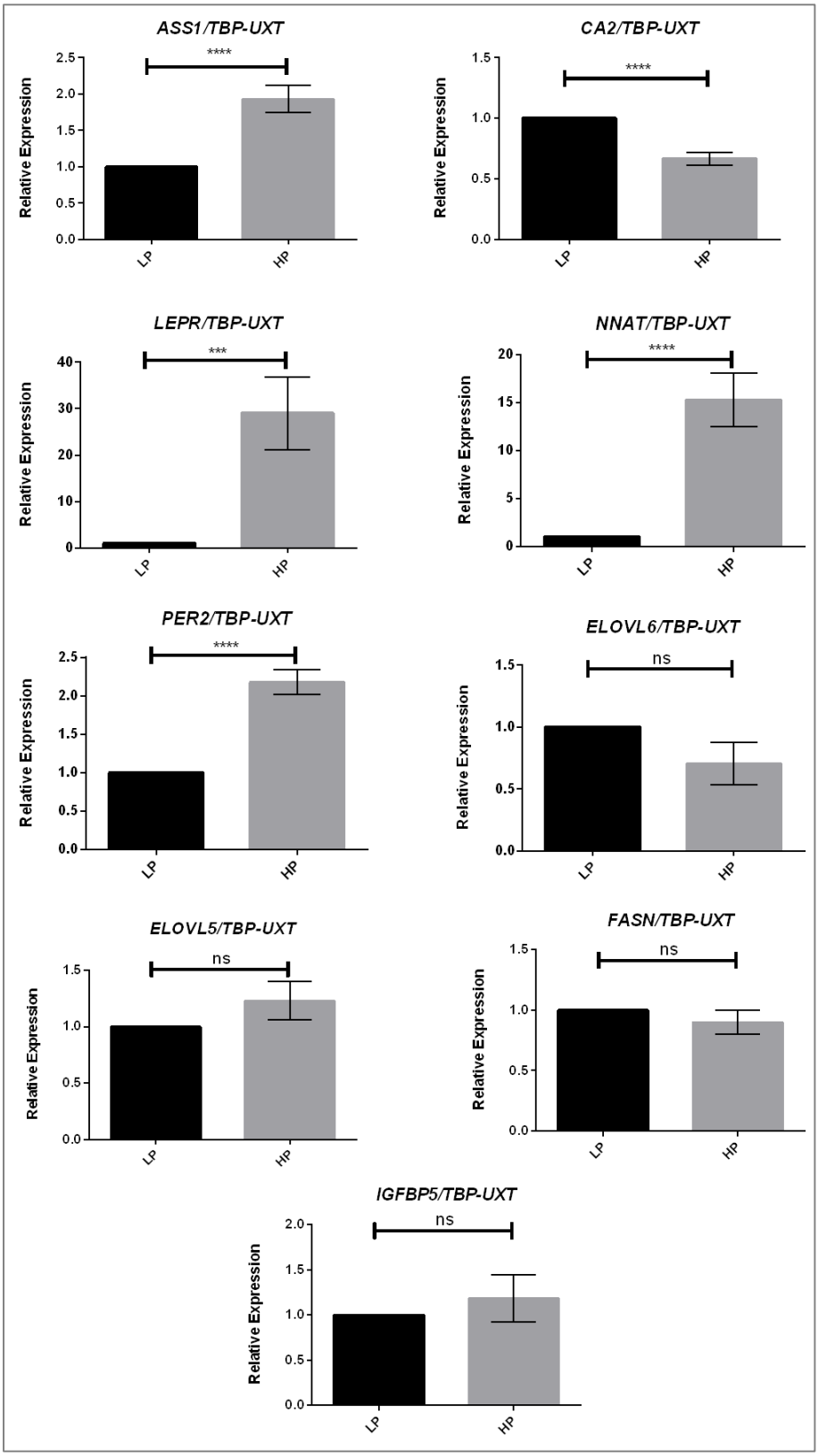


Figure 3.18. qRT-PCR validation of mRNA expression from peri-renal AT between LP and HP. Results from *ASS1*, *CA2*, *LEPR*, *NNAT*, and *PER2* were consistent with the data obtained from RNA-seq, but *ELOVL5*, *ELOVL6*, *FASN*, and *IGFBP5* were not . Mean values ± SEM. Level of significance: ns = non-significant, *** = $p < 0.0005$, **** = $p < 0.0001$. LP n = 6, HP n = 6.

3.3.2 Fatty acid composition of peri-renal adipose tissue

A total of 36 FAs were detected using GC (Table 3.5, 3.6 and 3.7), however only FAs with relative abundance > 0.1% were used for analysis: C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, and C22:0 (SFA); C14:1, C16:1, C17:1, C18:1n9t, C18:1n9c, C20:1 (MUFA); C18:2n6t, C18:2n6c, and C18:3n3 (PUFA).

3.3.2.1 Comparison between Holstein and Hereford

3.3.2.1.1 Analysis of fatty acid profiles

Breakdown of the FA categories showed that SFAs were the largest component in AT of both Holsteins (64.68%) and Herefords (66.31%), this was followed by MUFAs (33.56% in Holsteins, 31.67% in Herefords), and PUFAs were at relatively minute amount comparatively (1.75% in Holsteins, 2.02% in Herefords); (Figure 3.19). The SFAs and PUFAs abundance were lower in Holsteins compared to Herefords, by 1.83% and 0.26% respectively, while MUFAs was higher in Holsteins by 1.90% compared to Herefords. This also meant that SFAs:MUFAs ratio is higher in Herefords compared to Holsteins.

Figure 3.20 showed that majority of SFAs were palmitic (C16:0) and stearic (C18:0) acids, with an average total of 55.48% in Holsteins and 57.12% in Herefords. Among SFAs, the concentration of pentadecylic acid (C15:0) was significantly different between the breeds, 0.40% in Holstein versus 0.72% in Hereford (FDR < 0.05). The highest amount of MUFAs was oleic acid (C18:1n9c). The average total of oleic acid for Holsteins was slightly higher at

30.09%, while it was 28.03% in Herefords. However, the composition of MUFAs were not found to be statistically differently between the breeds. The PUFAs content was generally low in both breeds, with the highest FA, linoleic acid (C18:2n6c), at 0.99% and 1.29% for Holsteins and Herefords respectively. This PUFA was also one of the three FAs that were statistically different between the breeds. Another PUFA that was statistically different in composition was linolenic acid (C18:3n3), its composition was higher in Herefords at 0.44% compared to 0.18% in Holsteins.

3.3.2.1.2 Hierarchical cluster analysis

Hierarchical clustering compares the FA composition of peri-renal AT of Holstein and Hereford cattle as determined through GC. From a total of 36 FAs detected through GC, these were filtered down to 29 with at least 1 observation with an absolute value of $\geq$ 1.0, and difference between maximal and minimal values $\geq$ 1.0. Results showed distinctive separation of the breeds at the extreme ends on both side, however there was a mix of 2 breeds at the central region of the heat map (Figure 3.21). Therefore, FA profiles were unable to effectively differentiate the breeds.

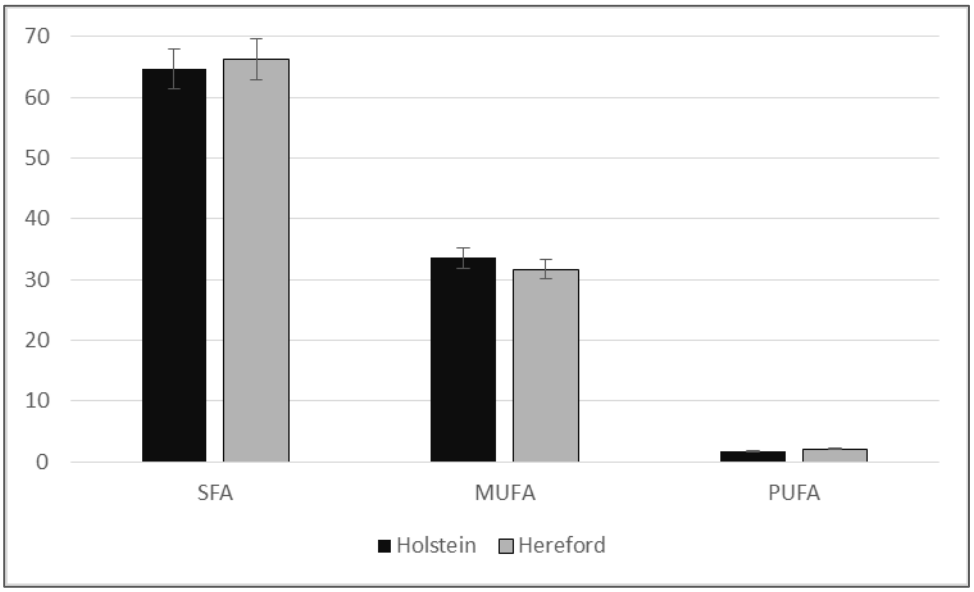


Figure 3.19. Comparison of FA categories between Holstein and Hereford cattle. FAs were categorised into SFA, MUFA, and PUFA. SFAs and MUFAs made up almost the entire composition of FAs in peri-renal AT of both breeds, with SFAs forming the largest component, followed by MUFAs. Holstein has a slight lower percentage of SFAs but higher percentage of MUFAs compared to Hereford. PUFAs were at a relatively minute and similar percentages between the two breeds. Mean values ± SEM. Holstein n = 6, Hereford n = 6.

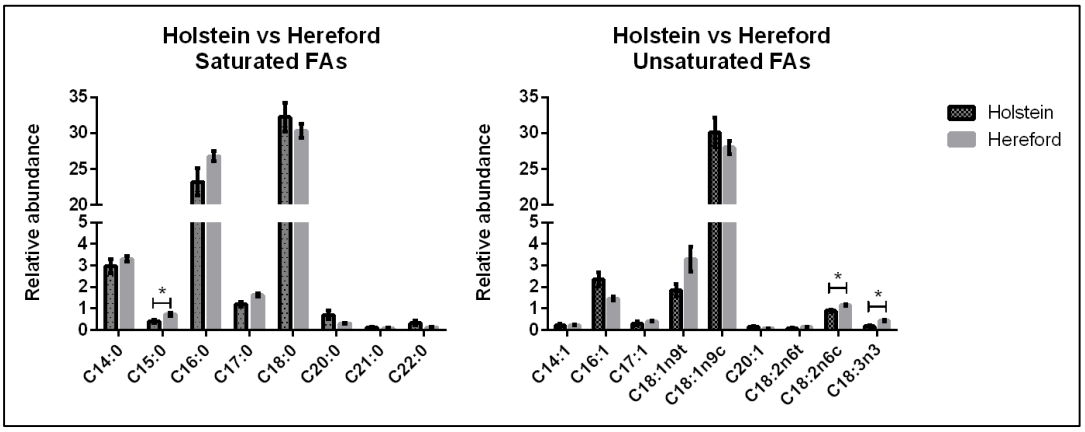


Figure 3.20. Comparison of FAs relative abundance from peri-renal adipose tissue of Holstein and Hereford cattle. Palmitic acid (C16:0) and stearic acid (C18:0) were the most predominant SFA in both breeds, while oleic acid (C18:1n9c) was the highest abundant unsaturated FA. Multiple t-test showed significant differences in pentadecylic acid (C15:0), ω-6 linoleic acid (C18:2n6c), and ω-3 linolenic acid (C18:3n3) with FDR < 0.05. Mean values ± SEM. Holstein n = 6, Hereford n = 6.

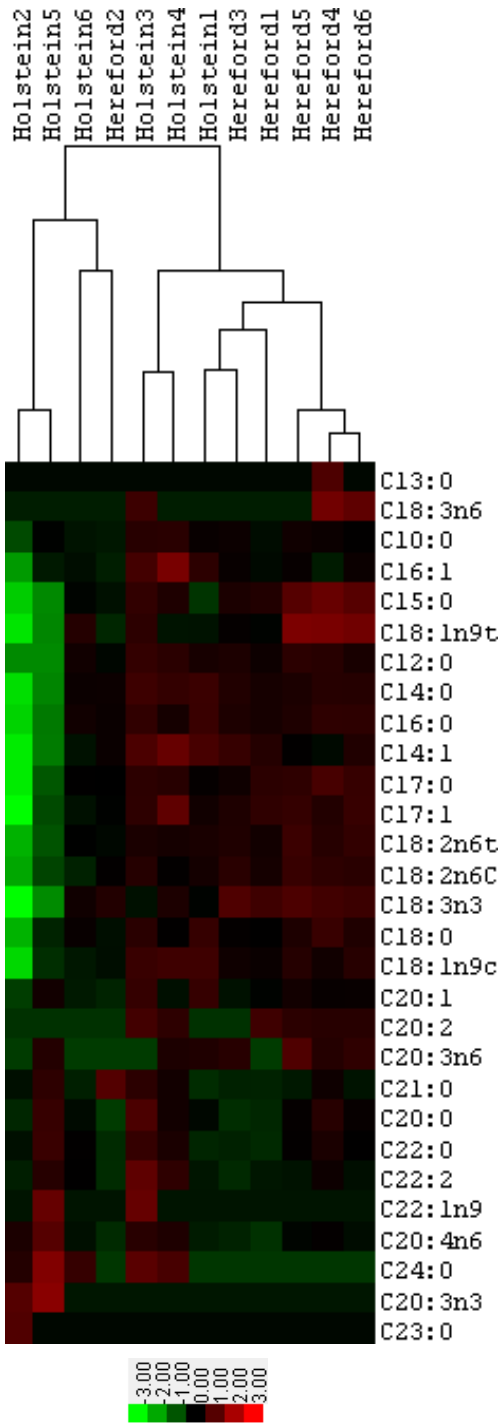


Figure 3.21. Hierarchical clustering of FA detected through gas chromatography in peri-renal AT of Holstein, and Hereford cattle. From a total of 36 FAs detected through GC, these were filtered down to 29 with at least 1 observation with absolute value of 1.0, and difference between maximal and minimal values $\geq$ 1.0. By comparing GC results from Holsteins and Herefords, FA composition between these 2 breeds appeared to exhibit some overlaps. Holstein n = 6, Hereford n = 6.

3.3.2.2 Comparison between LP and HP

3.3.2.2.1 Analysis of fatty acid profiles

As shown in the composition of FAs between peri-renal AT of Holstein and Hereford cattle, SFAs were the main component of FAs in LP and HP, at 63.09% and 62.42% respectively (Figure 3.22). The MUFAs had the next highest composition (34.74% in LP and 35.38% in HP), approximately half of that of SFAs, and PUFAs only made up a relatively small quantity of slightly over 2% (2.17 in LP, 2.19% in HP). However, the composition breakdown between the 2 treatments were fairly similar, with a difference of 0.67% in SFAs, 0.65% in MUFAs and only 0.02% in PUFAs.

Similarly, palmitic (C16:0) and stearic (C18:0) acids were the predominant FAs between AT of LP and HP, as were between the Holsteins and Herefords, the average total was 56.04% in LP, and 54.97% in HP. Oleic acid (C18:1n9c) was the most abundant MUFA, with almost identical amount of 30.37% and 30.96% for LP and HP respectively. The highest percentage of PUFA was linoleic acid, and its composition was the only one found to be statistically different between AT of LP and HP (FDR < 0.05). The relative abundance of linoleic acid was 1.15% in LP, and 1.51% in HP (Figure 3.23).

3.3.2.2.2 Hierarchical clustering analysis

Hierarchical clustering was used to display a global view of FA profiles in peri-renal AT of LP and HP. From a total of 36 FAs detected, these were filtered down to 23 FAs with at least 1 observations with absolute value of $\geq$ 1.0, and

difference between maximal and minimal value $\geq$ 1.0. Results showed that clusters were formed of both LP and HP, this suggests that dietary protein levels did not significantly alter AT FA composition of these animals (Figure 3.24).

To aid in interpreting these results, grades of carcass conformation, fat content and dead weight of these animals were compared, as detailed in Table 3.16. Samples were arranged in accordance with hierarchical clustering in Figure 3.24. Conformation and fatness grading of carcass from EC Reference Specification and UK Specification were used by the abattoir (AHDB, 2008). Conformation was divided to 5 main subjective classes: E, U, R, O and P, with classes U, O, and P further sub-divided into upper (+) and lower (-) bands. Conformation class E described carcases of outstanding shape, particularly of the type produced by heavily muscled cattle, such as Charolais and Belgian Blue, and Class P described poorly muscled carcases of inferior shape, such as those from cattle of high producing dairy cows. Fat content was assessed in 5 classes from 1 (very lean) to 5 (very fat); (AHDB, 2008). It appeared that the left cluster in the heat map, grouped animals with lesser muscling, higher level of fat deposition, and lower dead weight (Figure 3.24). Moving towards to right of the heat map, animals were of better conformation grade, leaner, and had higher dead weight.

Clustering analysis was conducted again on animals with fat class of 4, thus eliminating the possible influence of fat deposition level on the replicates. Results illustrated clustering was correlated with carcass conformation (Figure 3.25), with animals of poorer conformation clustered towards the left of the

heat map, while the ones with better conformation score were at the right end of the heat map (Table 3.17). Therefore, it appears that samples were clustered on the basis of conformation score independent of dietary protein treatment group.

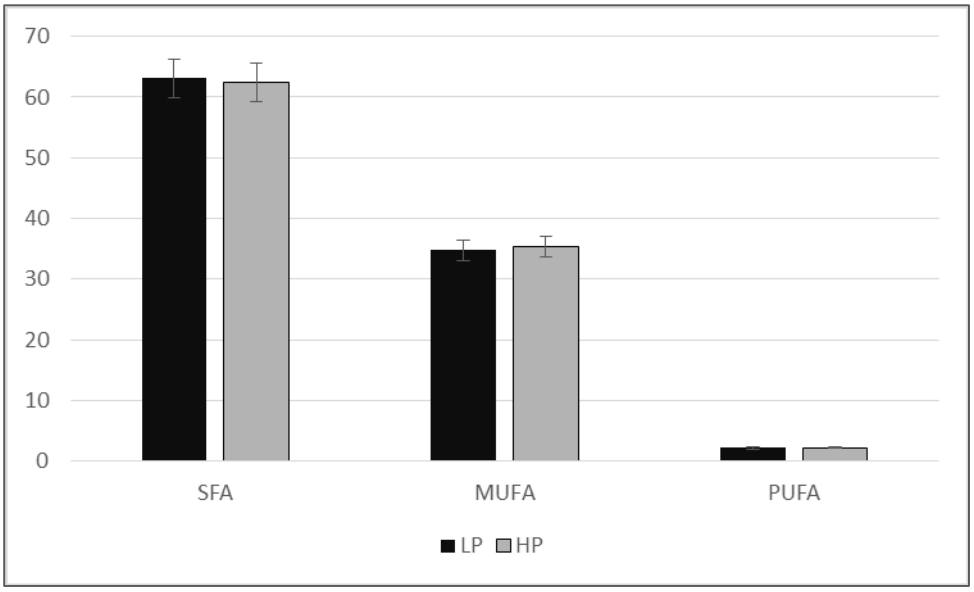


Figure 3.22. Comparison of FA categories between LP and HP. FAs were categorised into SFA, MUFA, and PUFA. SFAs and MUFAs made up almost the entire composition of FAs in peri-renal AT of LP and HP, with SFAs forming the largest component, followed by MUFAs, and a comparatively small amount of PUFAs. Percentages of SFAs and MUFAs differed by less than 1% between the diets, with the difference between LP and HP in PUFA was only 0.2%. Mean values ± SEM. LP n = 6, HP n = 6.

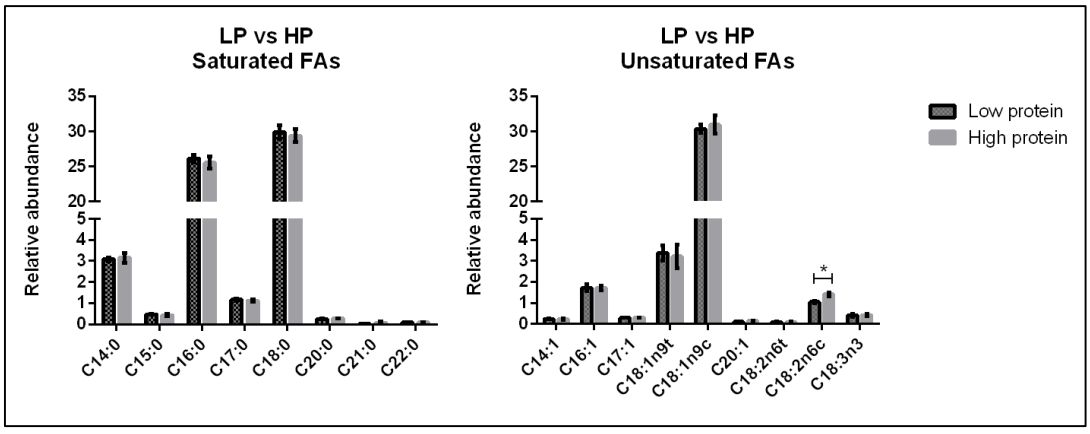


Figure 3.23. Comparison of FAs relative abundance from peri-renal adipose tissue of LP and HP. Palmitic acid (C16:0) and stearic acid (C18:0) were the most predominant SFA in both breeds, while oleic acid (C18:1n9c) was the highest abundant unsaturated FA. Multiple t-test showed significant differences only in ω-6 linoleic acid (C18:2n6c) at FDR < 0.05. Mean values ± SEM. LP n = 6, HP n = 6.

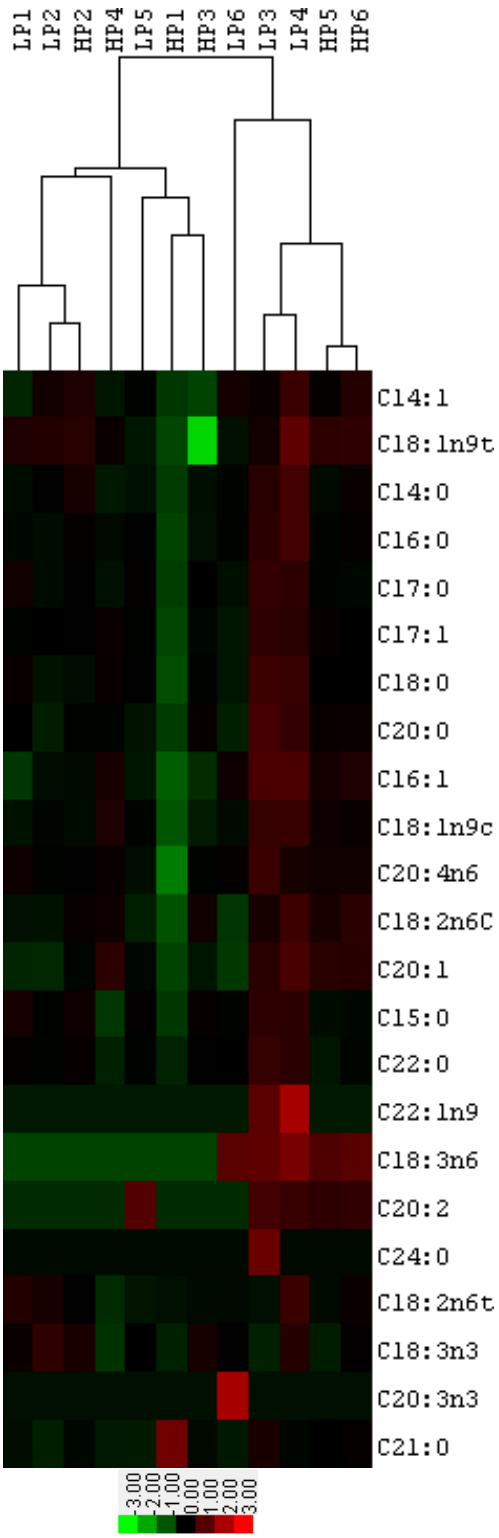


Figure 3.24. Hierarchical clustering of FAs detected through gas chromatography in peri-renal AT of LP and HP. From a total of 36 FAs detected, these were filtered down to 23 FAs with at least 1 observations with an absolute value of 1.0, and difference between maximal and minimal values $\geq$ 1.0. Results showed clusters were formed of animals fed on LP and HP, this may suggest dietary protein levels did not altered AT FA composition of these animals. LP n = 6, HP n = 6.

Table 3.16. Age, carcass conformation, fat class and dead weight of LP and HP. Samples were arranged in accordance to hierarchical clustering in Figure 3.24, with top row in this table referring the most left column of the heat map. EC Reference Specification and UK Specification standard grading system for conformation and fatness of carcass were used. Conformation was divided to five main classes: E, U, R, O and P, with E as the highest grade. Classes U, O, and P were further sub-divided into upper (+) and lower (-) bands. Fatness was assessed in 5 classes from 1 (very lean) to 5 (very fat). Animals with poorer confirmation, higher fatness grade, and lower dead weight appeared to the left of heat map, regardless of dietary protein level.

ID	Age/months	Conformation	Fat Class	Dead weight/kg
LP4	28	O	4	262.0
LP6	28	O+	4	291.8
HP6	28	O	4	278.5
HP12	28	O+	4	277
LP39	28	R-	4	300.6
HP4	26	O	4+	266.4
HP7	28	O+	4	286.2
LP41	28	O	4	267.9
LP8	28	O	4	327.9
LP11	25	O+	4-	304.6
HP14	25	O+	4-	321.3
HP15	28	O+	3+	269.7

Table 3.17. Age, carcass conformation, fat class and dead weight of LP and HP with fat class 4. Samples were arranged in accordance with hierarchical clustering in Figure 3.25, with top row in this table referring the most left column of the heat map. EC Reference Specification and UK Specification standard grading system for conformation and fatness of carcass were described in previous table. Animals with poorer conformation score were clustered towards the left of the heat map, while animals with better conformation were more towards the right.

ID	Age/months	Conformation	Fat Class	Dead weight/kg
LP8	28	O	4	327.9
HP6	28	O	4	278.5
LP4	28	O	4	262.0
LP6	28	O+	4	291.8
LP41	28	O	4	267.9
LP39	28	R-	4	300.6
HP12	28	O+	4	277
HP7	28	O+	4	286.2

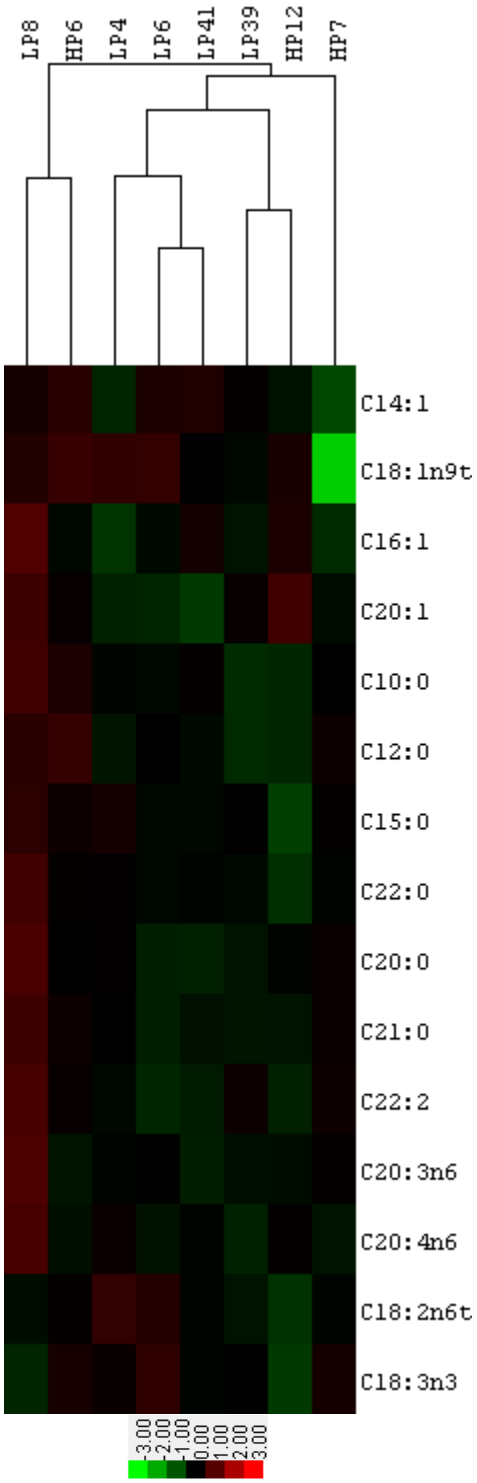


Figure 3.25. Hierarchical clustering of FAs detected through gas chromatography in peri-renal AT of LP and HP with fat class 4. From a total of 36 FAs detected, these were filtered down to 15 FAs with at least 1 observations with absolute value of 1.0, and difference between maximal and minimal values $\geq$ 1.0. Results showed that animals on different dietary protein treatment were not distinctively segregated, and clusters formed were made up of AT samples from animals on either diet. LP n = 5, HP n = 3.

3.4 Discussion

3.4.1 Animals and samples

The age of the dairy cows (Holstein) was relatively older than the beef cattle (Hereford) as sample collections were opportunistic and the Holsteins were post-production animals, while the Herefords were heifers that were meant to be slaughtered for meat. Although this situation may not be ideal, all animals were sexually matured and within their production life span, thus were still deemed viable to produced relevant comparative results. For FA profiling, samples were selected from animals of similar ages in order to reduce variability between samples within each treatment group. This was possible as more samples were available at the time as FA extraction was performed at a later stage compared to RNA-seq.

Sample collections were carried out throughout the year, as sampling was opportunistic and the availability of suitable animals were unpredictable, or dependent on the farm operations. Therefore, seasonal effects between treatment groups may be present, although these cannot be determine as samples collected for each treatment group were not spread out across all seasons. However, to minimise the effect of seasonal changes, samples within each treatment groups selected for qRT-PCR and FA profiling were collected within the same season.

3.4.2 Identification of differentially expressed genes

RNA-seq was used in preference to microarray in this study as it provides an unbiased assessment of all transcripts with a greater dynamic range, where quantification of expression levels would not be limited to a range of signal intensities as in microarray (Wang et al., 2009). Moreover, commercial microarray chip gene composition currently available are based on outdated reference genome which was released in 2009 (Agilent, 2015a). While with RNA-seq, the current *Bos taurus* genome assembly released in December 2009 and updated in September 2011, produced by Center for Bioinformatics and Computational Biology (CBCB) at University of Maryland (UMD3.1, November 2009), was used in this study (Ensembl, 2015a).

The number of raw reads of Hereford samples were over 20 million less than that of Holsteins (Table 3.8). This was because Holstein samples were sequenced twice in order to generate sufficient reads specified in contract with Edinburgh Genomics. However, the lower read numbers of Hereford samples would not have affected the results of this study. Firstly, read numbers adhered to the minimal criteria stated in Standards, Guidelines and Best Practices for RNA-seq v1.0 released by the Encyclopedia of DNA Elements (ENCODE) Consortium (ENCODE, 2011), where a minimal of two biological replicates were used, with 30 million paired-end reads of length over 30 nucleotides, of which 20 - 25 million reads are mappable to the genome or known transcriptome. Secondly, reads from both Holstein sequences were pooled and normalised against the total read counts, as were the reads from the

Herefords. Thus, expression values in Holsteins would not be higher due to the greater read numbers.

The edgeR software (www.bioconductor.org/packages/release/bioc/html/edgeR. html) was used for analysis of DEG as it is recognised as robustly designed and well-documented software which can account for biological variability of studies with only one or two replicate samples (Robinson et al., 2010). The pipeline used for RNA-seq analysis was to map quality controlled reads to the reference genome using Tophat2 (as a wrapper for Bowtie2), HTSeq was used to convert sequence reads to counts, and to identify significantly DEG using edgeR. The edgeR software uses empirical Bayes estimation and exact tests based on the negative binomial distribution to identify statistically robust DEG (Robinson et al., 2010; Zhang et al., 2014). The use of FDR as criteria over p-value was because FDR calculates the percentage of false positives within significant results that was generated using p-values in multiple comparisons such as in RNA-seq. Therefore, this will reduce the incidence of rejecting a true hypothesis (Benjamini and Hochberg, 1995). Also, the cut off criteria imposed by using FDR is less conservative that the Bonferroni approach in rejecting a hypothesis, thus FDR-controlled procedures have a greater power to discover significant results (Benjamini and Hochberg, 1995).

3.4.3 Gene ontology annotation

The GO term enrichment annotation was used to generate a general overview of biological processes and molecular functions that were overrepresented in

DE genes (Rhee et al., 2008) between AT of cattle breeds, and between dietary protein levels.

3.4.3.1 Holstein versus Hereford cattle

The GO indicated higher adiposity and adipogenesis in Herefords compared to Holsteins through higher mRNA expression of the genes reported to be involved in lipid metabolism (Table 3.9). The following discussion is on genes with higher expression levels in the Herefords compared to the Holsteins. Expression levels of genes related to adipogenesis and lipogenesis in other species appear to supports GO of DEG between Holsteins and Hereford. Higher mRNA abundance of insulin-induced gene 1 (*INSIG1*) was reported in AT of pig breeds with higher adiposity and rats fed on high-fat diet (Li et al., 2002, Liu et al., 2012b). Furthermore, *Insig1* mRNA progressively increased when 3T3-L1 preadipocytes were induced to differentiate into adipocytes (Li et al., 2003). Agouti signalling protein (*ASIP*) appeared to be related to high adiposity, this was shown through higher *ASIP* mRNA expression in Japanese Black (JB) steers with greater amount of intramuscular fat (IMF) compared to Holstein steers (Albrecht et al., 2012). ATP-citrate lyase (*ACLY*) was expressed particularly highly in tissues with high levels of lipogenesis, and was reported to be responsible for synthesis of acetyl-CoA, an essential building block for cholesterol and triglycerides (Sun et al., 2010, Wellen et al., 2009). RNA binding motif protein 38 (*RBM38*) was shown to maintain fat mass in mice where *Rbm38*$^{-/-}$ mice exhibited significant reduction of AT and smaller adipocytes compared to the wildtype (Zhang et al., 2014a). S100 calcium

binding protein A8 (*S100A8*) mRNA was highly expressed in differentiated 3T3-L1 adipocytes, and in AT of obese mice compared with lean mice (Sekimoto et al., 2012). Furthermore, significant increase of *S100A8* was detected in adipocytes of mice on high fat and high sucrose diet (Sekimoto et al., 2015). This was also observed in people where higher mRNA expression was recorded with increased visceral and subcutaneous fat mass (Sekimoto et al., 2012).

Meanwhile, the mRNA expression of the following genes was higher in Holsteins compared to Herefords. Interferon regulatory factor 4 (*IRF4*) have been reported to induce lipolysis in adipocyte through expression of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL); (Eguchi et al., 2008, Eguchi et al., 2011). Intracerebroventricular (i.c.v.) administration of neurotensin (*NTS*) inhibited feeding in rats (Luttinger et al., 1982, Levine et al., 1993). Furthermore. *LEP* was reported to influence levels of *NTS* mRNA and protein expressions, both found decreased in LEP-deficient ob/ob mice and i.c.v. administration of LEP stimulates expression of *NTS* mRNA in the hypothalamus (Wilding et al., 1993, Sahu, 2003). NADPH oxidase 4 (*NOX4*) was found to have anti-adipogenic role where NOX4-deficient mice display latent AT accumulation and are susceptible to diet-induced obesity and early onset of insulin resistance (Li et al., 2012b). Ryanodine receptor 3 (*RyR3*) was expressed abundantly in 3T3-L1 cells, although its level decreased upon induction of adipogenesis, while *PPARγ* protein level was markedly increased. Moreover, qRT-PCR analysis demonstrated that adipocytes expressed lower *RyR3* compared to stromal cells from mice AT (Tsai et al., 2013).

Mitotic checkpoint serine/threonine kinase B (*BUB1B*) mRNA expression was higher in Hereford compared to Holstein cattle. *Bub1b* was found to be markedly decreased in ovaries of aged wildtype mice, and was suggested to be a possible cause of female infertility and birth defects in older animals, due to disruption of chromosome segregation in mitosis and meiosis during oogenesis (Baker et al., 2004). Higher expression in Herefords may indicate that these animals were less likely to experience fertility issues than Holstein cows. However, as both Holstein AT samples used for RNA-seq were from animals older than the ones from Herefords, lower expression in Holstein samples may also be due to age instead of the breed factor (Table 3.8). Nevertheless, detection of *BUB1B* mRNA expression in bovine AT may be of interest and further exploration could be carried out to identify if *BUB1B* in AT could be a fertility indicator in cattle.

Secreted phosphoprotein 1 (*SPP1*) was found to play a positive role in implantation and placentation in human, rats, sheep and pigs (Johnson et al., 2003, Erikson et al., 2009). Furthermore, expression of *SPP1* was reported to promote placenta efficiency in hyperprolific LW and MS sow, thus increasing the litter size (Fernandez-Rodriguez et al., 2011, Hernandez et al., 2013, Hernandez et al., 2014). Quantitative trait loci studies showed that *SPP1* may influence milk yield and composition in dairy cows (Cohen-Zinder et al., 2005, Leonard et al., 2005, Khatib et al., 2007, Dudemaine et al., 2014). Thus, it would be of interest to investigate if *SPP1* expression in cattle AT would be indicative of enhanced reproductive performance. However, further validation of this gene was not pursued in the current study as individual count reads

were not consistent higher in both Hereford samples compared to the Holsteins.

3.4.3.2 LP versus HP

GO of DEG in LP and HP suggests a trend towards higher adiposity and adipogenesis in LP. Among the top 25 genes expressed at a higher level in HP compared to LP, a number of these genes were anti-adipogenic as described below. Expression of *LEPR* is highest in the central nervous system, particularly the hypothalamus, although *LEPR* mRNA was also detected in various tissues including the AT (Ren et al., 2002, Chelikani et al., 2003). This suggests localised LEP activities in AT as shown by depletion of fat mass through the lipolytic action of LEP on rat adipocytes (Wang et al., 1999b). *In vitro* studies using mouse embryo fibroblasts (MEFs) and 3T3-L1 preadipocytes revealed that period circadian clock 2 (*Per2*) significantly repressed *Pparγ* transcription, thus reducing adipogenesis (Grimaldi et al., 2010). Carbonic anhydrase III (*CA3*) mRNA expression was shown to decrease adipogenesis, demonstrated by increased formation of cells with lipid deposition from *CA3* knockout MEFs together with increased *PPARγ* gene expression (Mitterberger et al., 2012).

Both fatty acid synthase (*FASN*) and stearoyl-CoA desaturase (*SCD*) had lower mRNA expression in HP compared to LP. *FASN* was found to induce lipogenesis through *de novo* biosynthesis of long-chain FAs from acetyl-CoA and malnoyl-CoA (Smith, 1994, Smith et al., 2003, Chirala and Wakil, 2004, Wang et al., 2004), and significant reduction in fat mass were discovered in

mice with *Fasn* knockout (Chakravarthy et al., 2005). Furthermore, *SCD* was shown to catalyse synthesis of unsaturated FA by insertion of a cis-double bound in the Δ9 position of FA substrate (Kim and Ntambi, 1999), and SCD knockout mice exhibited a reduction in body fat and resisted diet-induced weight gain (Ntambi et al., 2002).

By contrast, neuronatin (*NNAT*) mRNA expression was higher in HP. This gene was previously found to facilitate adipogenesis in rats, promote preadipocyte differentiation in 3T3-L1 cells, and increase transcription of *C/ebp* members involved in adipogenesis and induction of *Pparγ* (Suh et al., 2005a, Suh et al., 2005b). This also correlated with higher mRNA expression of *UCP1* in LP, as lower *Nnat* expression in murine primary adipocyte cultures showed elevated *Ucp1* expression, indicative of enhanced FA oxidation and lipolysis as *UCP1* has been reported to induce lipid metabolism (Gburcik et al., 2013).

3.4.4 Transcriptomic comparison

In previous sections of this chapter, genes and nutritional compounds that could affect adipogenesis and lipogenesis of AT in cattle have been outlined. From RNA-seq results, genes of interest were identified from the list of DEG between AT of Holstein (dairy breed) and Hereford (beef breed): *ELOVL5*, *INSIG1*, *IRF4*, *LDLR*, *LEPR*, *RAN*, *RXRα*, *SCD*; also between AT from AAX cattle fed different dietary protein levels: *ASS1*, *CA2*, *ELOVL5*, *ELOVL6*, *FASN*, *IGFBP5*, *LEPR*, *NNAT* and *PER2*. In the following paragraphs, DE of these genes will be discuss in more details to demonstrate their possible roles

in inducing cellular and metabolic changes in AT, and ways they might potentially influence reproductive performance of cattle.

3.4.4.1 Transcriptomic comparison between breeds

The *LEPR* has been reported to mediate the physiological effects of *LEP*, particularly in appetite control and energy expenditure. *LEP* expression also reflected adipocyte size and fat mass in fully fed animals (Houseknecht and Portocarrero, 1998). Expression of *LEPR* is highest in the central nervous system, particularly the hypothalamus, but *LEPR* mRNA was also detected in AT, semitendinosus muscle, liver, spleen, testis, pituitary, and lung in cattle (Chelikani et al., 2003, Ren et al., 2002). The widespread distribution of *LEPR* in various peripheral tissues is suggestive of localised functions of *LEP*, such as lipid metabolism in AT (Chelikani et al., 2003). This was demonstrated by depletion of denervated fat pads in hyperleptinemic rats, indicating that the lipolytic action of leptin on adipocytes was not mediated by signals from the central nervous system (Wang et al., 1999b). Furthermore, LEP suppresses *ACACA* and *FASN* (two important enzymes that catalyse FA biosynthesis) expressions in rodent preadipocyte and adipocytes cultures, also increased expression of acyl CoA oxidase (*ACO*) and carnitine palmitoyl transferase (*CPT*) an enzyme involved in long chain FA oxidation (Bai et al., 1996, Wang et al., 1999a). These results indicate that LEP was directly regulating AT metabolism by both inhibiting lipogenesis and stimulating lipolysis (Fruhbeck, 2001). This peripheral LEP action appears to be conserved in cattle as higher *LEPR* expression in Holsteins compared to Herefords (Figure 3.8) may be one

of the reasons behind lower adiposity in Holsteins. This also may explain lower expression of *ELOVL5*, and *SCD* in Holsteins versus Herefords, as *Lep* has been reported to suppress expression of *Elovl5* and *Scd* in mice (Biddinger et al., 2006, Singh et al., 2009).

The FA elongation process involves the addition of two-carbon units to the carboxyl end of a FA using acetyl-CoA and malonyl-CoA as substrates (Wang et al., 2005). The family of ELOVL fatty acid elongases (*ELOVLs*) are FA elongase enzymes that catalyse the elongation process (Leonard et al., 2000, Jakobsson et al., 2006). Among the subtypes that have been identified thus far, *ELOVL5* is the most abundant elongase found in various tissues, with highest level detected in liver, adrenal gland and testis (Guillou et al., 2010, Tripathy et al., 2010). In rodents, it was reported that *Elovl5* was responsible for a majority of hepatic FA elongation. Therefore, it was proposed that ELOVL5 is capable of elongating a broad range of FA substrates, including SFAs, MUFAs and PUFAs (Wang et al., 2005). However, it appeared that a complex positive-negative feedback loop formed between *ELOVL5* and sterol regulatory element binding protein 1 (*SREBP1*), a lipogenic gene, and *ELOVL5* expression was suppressed by presence of elevated levels of n-3 and n-6 PUFAs (Guillou et al., 2010, Tripathy et al., 2014). Presumably the elongation process is conserved across tissues, lower transcriptional expression in Holsteins versus Herefords may indicate lower FA elongation activities in the Holsteins, as a result of lower amount of substrates available due to lesser fat mass and lower adiposity in Holsteins. Also, the fertility of *Elovl5*$^{-/-}$ female mice was observed to be compromised (Guillou et al., 2010).

Thus differences in *ELOVL5* expression in cattle may also partly explain lower fertility performances in Holsteins compared to Herefords.

The *SCD* gene encodes an enzyme involved in *de novo* lipogenesis, particularly in desaturation of MUFAs, palmitic and stearic acids, and the end product of this process is primarily palmitoleic and oleic acids (Kim and Ntambi, 1999). As the major composition of membrane phospholipids and triacylglycerol stores in differentiated 3T3-L1 adipocytes are palmitoleic and oleic acids (Kasturi and Joshi, 1982), increased mRNA expression of *SCD* indicated elevated lipogenic activities in adipocytes. Furthermore, in 3T3-L1 cells, *Scd* was reported to promote glucose utilization for FA synthesis (Yee et al., 2013). Martin and colleagues (1999) observed that *SCD* mRNA concentration peaked prior to a significant increase in lipogenesis and lipid filling of adipocytes in bovine AT. They proposed that elevated *SCD* mRNA expression occurred as preadipocytes exited the proliferative phase and entered terminal differentiation. Thus elevated *SCD* mRNA expression in Herefords may indicate that AT was more actively undergoing lipid filling compared to Holsteins.

Expression of *INSIG1* has been shown to reflect the differences in adiposity between breeds. Results from comparing adipocytes of leaner Landrace pigs and fattier Rongchang pigs showed higher adipocyte cell diameter and *INSIG1* mRNA abundance in the latter (Liu et al., 2012b). The AT of rats fed on high-fat diet also illustrated an increase of *Insig1* mRNA compared to the controls (Li et al., 2002). Furthermore, induction of 3T3-L1 preadipocytes to differentiate into adipocytes demonstrated progressive increase of *Insig1*

mRNA as measured by Northern blot (Li et al., 2003). However, from the same study, overexpression of *Insig1* was reported to limit deposition of triglycerides in mature adipocytes and blocked preadipocyte differentiation by restricting upregulation of *Pparγ2*, adipocyte protein 2 (*aP2*), and carbohydrate response element-binding protein (*ChREBP*) expression in the fat tissue of normal mice at the onset of diet-induced obesity (Li et al., 2003). This would suggest that *INSIG1* not only exerted a negative control function to prevent over distention of the adipocytes with lipid deposition, it also initiated the recruitment of adjacent preadipocytes to increase fat-storage capacity of the fat mass (Li et al., 2003). Higher *INSIG1* mRNA expression in Herefords compared to Holsteins appeared to agree with observations in other species, and suggests that AT of Herefords had a higher level of adiposity with greater capacity to expand compared to the Holsteins.

Low density lipoprotein receptor (*LDLR*) is known to be the target gene of sterol regulatory element binding (*SREBP*); (Yokoyama et al., 1993), and increased levels of dominant positive *SREBP1* mutant stimulates the expression of *Ldlr* genes in 3T3-L1 adipocytes in a dose dependent manner (Lay et al., 2002). The gene *SREBP1* is expressed in adipocytes and activates genes involved in FA and triglyceride synthesis, such as *ELOVL5* and *ELOVL6* (Shimomura et al., 1997, Moon et al., 2009). Mice fed on high fat and high sucrose diet exhibited higher *Ldlr* mRNA expression compared to control animals (Matsuzaka et al., 2007). Higher *LDLR* expression in Herefords compared to Holsteins indicates increased lipogenesis of adipocytes in the former. Although in this case, *SREBP1* mRNA expression were not

significantly different between the breeds (Figure 3.8). This suggest that elevation of *LDLR* mRNA expression was induced by other factors.

The RXRα is a nuclear receptor that forms heterodimeric complex with PPARγ and others (Graves et al., 1992, Choong et al., 2015). Therefore, RXR is involved in multiple signalling pathways, including pathways that regulate adipogenesis and lipogenesis in AT (Tontonoz et al., 1994, Tontonoz and Spiegelman, 2008). Ablation of *Rxrα* in mice resulted in adipocytes that are resistant to dietary- and chemically induced obesity, thus demonstrating that *RXRα* is essential in adipocyte hypertrophy (Imai et al., 2001, Metzger et al., 2005). Increased numbers of small adipocytes in these mice also suggest that *RXRα* is involved in the preadipocytes differentiation process (Metzger et al., 2005). This was confirmed by Hamza et al. (2009) using paired-end tagging technology coupled with chromatin immunoprecipitation assays (ChIP-PET) to map PPARγ binding sites in 3T3-L1 preadipocyte cells, where RXR was observed to be the predominant heterodimeric partner for PPARγ during adipocyte differentiation and maturation. The mRNA expression of *RXRα* in Herefords was elevated compared to Holsteins in RNA-seq, and although a similar trend was observed by qRT-PCR, the difference was not statistically significant. Furthermore, *PPARγ* mRNA was not differentially expressed between the breeds (Figure 3.8). This may imply that differences in adiposity between breeds in this study inclined towards lipid filing of existing mature adipocytes rather than differentiation of preadipocytes, as reports showed that *PPARγ* expression was vital during preadipocyte differentiation phase in AT development (Tontonoz et al., 1994, Barak et al., 1999).

Ras-related nuclear protein (*RAN*) is a small GTP binding protein belonging to the RAS superfamily, and was found to be a coactivator to androgen receptor (Hsiao et al., 1999, Sen et al., 2014). *RAN* was also observed to be of importance for meiotic and mitotic spindle assembly in the mouse and *Xenopus* (Moore, 2001, Dumont et al., 2007). *RAN* protein expression was also detected in human placental tissue, although the physiological function of *RAN* was not characterised, it was proposed that its expression could be important for placental development during gestation (Kuyznierewicz and Thomson, 2002). Expression of *RAN* in AT has not been described thus far. If the functions described above can be translated to AT in cattle, higher *RAN* mRNA expression in Herefords would support the hypothesis that greater adiposity in this breed may exert an advantageous effect over Holsteins in terms of fertility.

Interferon regulatory factor 4 (*IRF4*) was reported to exert lipolytic effects in AT (Eguchi et al., 2008). *In vitro* studies using 3T3-L1 and *Irf4*$^{-/-}$ adipocytes revealed that lipolysis was diminished in adipocytes lacking *IRF4*, while ectopic *IRF4* overexpression enhanced lipolysis. This was attributed to its transcriptional influence on *ATGL* and *HSL*, enzymes involved in hydrolysis of triglyceride to free fatty acids (FFAs); (Kraemer and Shen, 2002, Zimmermann et al., 2009), where both mRNA and protein levels were reduced in the absence of *IRF4* (Eguchi et al., 2011). Also *IRF4* appear to inhibit lipogenesis as exogenous *IRF4* depressed expression of lipogenic genes such as *FASN*, *SCD*, and *SREBP1* in cell cultures (Eguchi et al., 2011). Furthermore, it was found to induce greater energy expenditure and cold tolerance through enhanced thermogenic gene expression, such as *UCP1* and peroxisome

proliferator-activated receptor gamma, coactivator 1 alpha (*PPARGC1α*); (Kong et al., 2014). Higher mRNA expression of *IRF4* in Holsteins may underpin the lesser degree of adiposity in this breed compared to Herefords, where lipids were heavily mobilised from adipocytes to sustain the high energy demand of milk production.

Overall, mRNA expression from RNA-seq results from this study indicated that AT of Holsteins had a lower expression of a number of important adipose-related genes compared to that of Herefords. The results obtained here also suggest that such difference may contribute to the superior fertility of Herefords which may be related to the availability of, and ability to exploit, energy reserves from their AT.

3.4.4.2 Transcriptomic comparison between dietary protein levels

Argininosuccinate synthase (ASS1) is a cytosolic enzyme that catalyses formation of arginino-succinate from citrulline and aspartate in the urea cycle during breakdown of protein molecules (Hampton et al., 2013). This step is crucial in metabolism and removal of nitrogenous waste as deficiency in *ASS1* resulted in citrullinemia, over accumulation of citrulline in circulation, and in severe cases lead to fatal neonatal hyperammonemia (Ah Mew et al., 1993, Engel et al., 2009). Expression of *ASS1* dropped significantly in the BAT of thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) during hibernation. As animals ceased feeding during hibernation, stored lipid was consumed at high rates by BAT to generate heat. Thus, it was proposed that low expression of *ASS1* during this period may indicate that the urea cycle was suppressed

(Hampton et al., 2013). If the above observation is conserved in WAT of cattle, decreased mRNA expression of *ASS1* in LP may indicate that breakdown of protein molecules was lower compared to HP due to the amount of protein ingested. This may also indicate that the differences in protein levels between treatment groups was sufficient to trigger transcriptomic changes in relation to protein metabolism.

Carbonic anhydrase II (CA2) is one of the carbonic anhydrase isozymes involved in catalysing the reversible hydration of carbon dioxide into bicarbonate (Sly and Hu, 1995). *De novo* lipogenesis was reported to be inhibited in rat hepatocytes treated with carbonic anhydrase inhibitors, trifluoromethylsulphanomide and ethoxozolamide (Lynch et al., 1995). It was proposed that carbonic anhydrase inhibition reduced conversion of pyruvate to CO_2, glyceride-glycerol, and FAs significantly (Bray, 1972), as carbonic anhydrase activity is required for optimal activity of pyruvate carboxylase (PC); (Lynch et al., 1995), an enzyme crucial in *de novo* FA biosynthesis (Jitrapakdee et al., 2008). Also, *CA2* protein expression was observed in differentiated and mature adipocytes of murine 3T3-L1 and 3T3-F442A, but was not detected during the preadipocyte stage (Lynch et al., 1993). On the other hand, skeletal muscle in Korean steers with higher IMF scores expressed a lower *CA2* protein level, yet the mRNA expression remained unchanged. The authors hypothesised that lower *CA2* expression in muscle may be due to IMF deposition (Zhang et al., 2010). Therefore, it was of interest to explore the effects of *CA2* on adipocyte and myocyte development. Using BEFS-PPARγ2 (BEFS = bovine embryonic fibroblasts) cell line, a bovine adipogenic model system, researchers recorded significant decreased in *CA2*

mRNA expression after adipogenesis, while *PPARγ* expression was significantly higher. Concomitantly, the myogenic BEFS-MyoD cell line illustrated an increased trend in *CA2* expression after myogenesis, although the increment was not statistically significant (Zhang et al., 2010). Thus the evidence suggests that *CA2* could be involved in the initiation stage of adipogenesis, whereas *PPARγ* functions to maintain the adipogenic phenotype of the differentiated cells. The mRNA expression of both *CA2* and *PC* (log FC HP vs LP = -0.78, FDR = 0.23) were elevated in LP compared to HP, this this may indicate that AT of LP incurred a higher level of adipogenesis and greater number of adipocytes compared to HP (Figure 3.26).

Global gene expression profiling by cDNA microarrays previously showed that *Nnat* had significantly increased expression in 6-week old Zucker diabetic fatty (ZDF) rats compared with age- and sex- matched Zucker lean control (ZLC) rats. However, the expression level was reversed when these animals were 20-weeks old (Suh et al., 2005b). This indicated that *Nnat* may facilitate adipogenesis. Further studies revealed that *Nnat* promotes preadipocyte differentiation in 3T3-L1 cells, mediated through increased transcription of *C/ebpβ* and *C/ebpδ*, which induce *Pparγ* expression that eventually lead to upregulation of *C/ebpα* (Suh et al., 2005a). Meta-analysis using 4 different datasets derived from microarray gene expression of mouse WAT showed conservation of transcription factor binding sites of genes involved in adipogenesis and lipogenesis in the *Nnat* promoter, including *Pparγ* and *Srebp1* (Li et al., 2010). Results from protein-protein interaction networks analysis using BioNetBuilder (http://err.bio.nyu.edu/cytoscape/bionetbuilder/) also showed that *Nnat* expression was positively correlated with early B-cell

factor 1 (*Ebf1*) and lectin, galactose binding, soluble 12 (*Lgals12*); (Li et al., 2010), both associated with proadipogenic *C/EBP* transcription factors, inducing adipocyte differentiation and lipogenesis (Akerblad et al., 2002, Yang et al., 2004, Jimenez et al., 2007, Yang et al., 2011, Gao et al., 2014). RNA interference (RNAi) silencing of *Nnat* expression in murine primary adipocyte cultures demonstrated increased expression of *Ucp1,* as well as *Ppargc1α*, key genes in mitochondrial oxidative phosphorylation, indicative of enhanced FA oxidation and lipolysis (Gburcik et al., 2013). Thus, higher *NNAT* mRNA expression in HP suggest that the fat mass had a greater degree of hypertrophy and hyperplasia as compared to LP. This is contradictory with the expectation that higher dietary protein level would reduce adiposity, as has been reported in humans and rodents previously. In fact, lipolytic effect of *LEP* described in previous section, may also have contributed to increase lipolytic activities in HP, as *LEPR* mRNA expression was significantly greater in HP compared to LP (Figure 3.26). Also of note, *Nnat* mRNA expression was positively correlated with protein turnover in mice, a measure of protein synthesis and degradation rate (Li et al., 2010). This suggest that higher dietary protein intake in HP stimulated activities of protein metabolic pathways due to the increase in protein availability.

Period circadian clock 2 (*PER2*) is a member of the Period family of genes involved in circadian homeostasis. Circadian rhythmicity occurs in virtually all mammalian systems in response to environmental changes, controlled by the endogenous clock in suprachiasmatic nucleus (SCN) of the anterior hypothalamus, with input from cells of peripheral tissues (Reppert and Weaver, 2002, Dibner et al., 2010). *In vitro* studies using MEFs and 3T3-L1

preadipocytes revealed that *Per2* repressed *Pparγ* binding to the PPAR responsive element (PPRE) of fatty acid binding protein 4 (*Fabp4*), thus significantly reduced *PPARγ*-dependent transcription, even in the presence of rosiglitazone, a *PPARγ* agonist (Grimaldi et al., 2010). In addition, both MEFs and 3T3-L1 preadipocytes with *Per2* knockdown through RNAi showed a greater number of preadipocytes differentiated into adipocytes compared with controls (Grimaldi et al., 2010). Conversely, depletion of *Per2* did not lead to an increase of lipid deposition in adipocytes of *Per2*-null mice, although an elevation of SFAs and MUFAs was observed using high performance liquid chromatography and mass spectrometry (Grimaldi et al., 2010). The increase of SFAs and MUFAs was attributed to induction of *Elovl3*, a *Pparγ* target gene that catalyses synthesis of SFAs and MUFAs (Westerberg et al., 2006). The mRNA expression and function of *PER2* in AT of cattle has not been previously reported. If the effects of *PER2* described above are conserved in bovine AT, lower *PER2* mRNA expression in LP would lead to increased level of adipogenesis and build-up of SFAs and MUFAs in adipocytes, compared to HP (Figure 3.26).

Other lipogenic genes with significant DE between LP and HP in RNA-seq were not validated by qRT-PCR, including *FASN*, *ELOVL6*, *ELOVL5*, and *SCD*. The gene expression of *FASN* was reported to induce *de novo* biosynthesis of long-chain FAs from acetyl-CoA and malnoyl-CoA, producing palmitic acid, a 16-carbon FA (Smith, 1994, Smith et al., 2003, Chirala and Wakil, 2004). Mice with *FASN* liver specific knockout (FAS LKO) showed significantly reduced fat pad weight compared to wild type mice, together with elevated levels of malonyl-CoA and decreased levels of palmitate acid in the

liver (Chakravarthy et al., 2005). A study in pigs fed a reduced protein diet showed increased IMF deposition, accompanied by raised *SCD* and *PPARγ* mRNA levels. A higher total FA content was also recorded, and the increase of SFAs was associated with the higher mRNA expression of *ACACA*, *C/EBPα*, *FASN* and *SCD* (Madeira et al., 2013). *ELOVL6* catalysed the elongation of 12-, 14- and 16-carbon FAs by adding 2 carbon molecules to the fatty acid chains in each step (Matsuzaka et al., 2002). Rats were fed on restricted diet (50% of the amount consumed by the control group) for 30 days, then half of these rats were fed *ad libitum* as the control rats for two days, while the rest were kept on the restricted diet. The inguinal, peri-renal and epididymal fat pads from rats on restricted diet for the entire study period were lighter, and had lower *Elovl6* expression compared to control rats. When rats on restricted diet were refed *ad libitum*, fat masses increased, together with elevated *Elovl6* expression. Simultaneously, gene expression of *Fasn*, *Scd*, and *Acaca* had similar pattern as *Elovl6* (Turyn et al., 2012). Furthermore, *ACACA* catalysed synthesis of malonyl-CoA from acetyl-CoA and mice deficient in *Acaca* experienced continuous FA oxidation thus led to loss of body fat and weight (Tong, 2005). In the current study, the mRNA expression of *ELOVL6* was higher in LP compared to HP, although this was not statistically significant. The mRNA expression of *ACACA* from RNA-seq results was also lower in HP compared to LP, with log FC of -1.16 (FDR < 0.05). This may implied that there was possible higher volume of malonyl-CoA, therefore possibly a higher rate of *de novo* FA biosynthesis in LP compared to HP.

Collectively, the data obtained here indicated that cattle on a low protein diet have a higher level of adipogenesis and lipogenesis related mRNA expression as compared to cattle on high protein diet, as evidenced by significant changes in transcriptomic expression of the genes involved in these processes. However the high and low protein diets did not induce any changes in mRNA expression of *PPARγ* and *C/EBPs,* both important regulators of adipogenesis. This may be due to the comparatively modest difference in protein levels (LP = 10%, HP = 14%), whereas dietary protein levels tested in humans and lab-rodent research have ranged from the lowest at 12% to a high of 36% (Parker et al., 2002, Westerterp-Plantenga, 2003, Stepien et al., 2011). Furthermore, the cattle were on low and high protein diets for only 8 weeks, in comparison to 12 to 27 weeks in humans. Previous studies in rats showed lipogenesis inhibition through significant reduced *Fasn* and *Acaca* mRNA expression from the 1st day of treatment, and depressed expression continued till observation was ended at day 14 (Stepien et al., 2011). However, the protein content was 3.7 times higher in the high protein group compared to the control group (Stepien et al., 2011). Therefore, it is possible that the dietary protein effects were not extensive in AT of the animals in the current study due to both the duration of feed intervention and the small differences in protein levels between diets. Moreover, the effects of dietary protein level in cattle may not be parallel to results from human and rodents as the latter are monogastric animals that could digest and absorb protein directly, while protein ingested by cattle (ruminants) goes through the biohydrogenation by ruminal microbes (Cecava, 1995b).

Digestibility of dietary protein provided in LP and HP was assumed to be similar, although this may not be the case as feed materials were different in each diet, plus the composition of the premix was not available due to commercial confidentiality. Therefore, the proportion of RDP and RUP, as well as NPN, may differ between the diets. Furthermore, protein ingested was degraded by ruminal microorganisms, and a considerable amount of the protein absorbed and utilised by the animal originated from digestion of microbial protein (Cecava, 1995b). All these could affect the actual amount of amino acids available to the animal and the quantity of energy used during metabolism of these materials.

It has been suggested that amino acid supplementation should be customised to provide only the specific essential amino acid deficient in the animal (Walker, 2012). The basis of this proposal was that an increase in dietary protein as a whole will increase the amount of non-essential amino acids to an excessive level. These non-essential amino acids would need to be metabolised, thus causing a loss of energy as protein metabolism is an energy expensive process. This would lead to a compromised feed conversion rate, thus undermining the value of protein supplementation and its potential to improve productivity (Walker, 2012).

Hence, determination of the biological value of a feed protein in ruminant can be complicated due to the biohydrogenation process, and direct comparison of the amount of amino acid ingested to the amount excreted from the gastrointestinal tract may not be a true indicator. One possible route would be to assess the amount and type of amino acid appearing in the portal circulation

of the animal through serum or plasma biochemistry analysis and gas-liquid chromatography, as this may represent the closest value of amino acid absorbed into various cells and tissues (van der Walt and Meyer, 1988, Lewis et al., 1980).

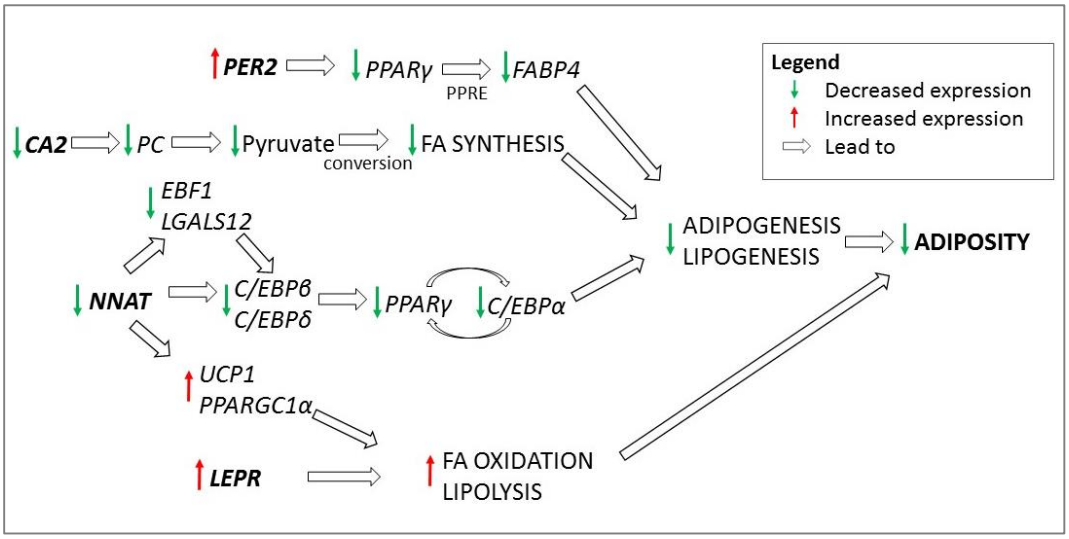


Figure 3.26. Proposed schematic model of mRNA expressions influencing adiposity between LP and HP. Genes of interest identified from DEG list between LP versus HP are *CA2*, *NNAT*, *LEPR* and *PER2*. Development of adiposity with inference of results from previous studies on genes of interest are illustrated. Higher expression of *PER2* lead to decreased binding of *PPARγ* to the PPRE of *FABP4* thus reduces *PPARγ*-dependent transcription. Lower expression of *CA2* suppressed activities of PC, therefore reducing conversion of pyruvate into FA. Lower expression of *NNAT* lead to lower adiposity through decreased adipocyte generation by direct lowering of *C/EBPβ* and *C/EBPδ* expression, or through reduced expression of *EBF1* and *LGALS12*, which in turn reduce expression of *PPARγ* and *C/EBPα*, important genes involved in both adipogenesis and lipogenesis. Also, lower *NNAT* expression increases FA oxidation and lipolysis through higher expression of *UCP1* and *PPARGC1α*. Similarly, increased *LEPR* expression also increased lipid metabolism and lead to reduction of adiposity. In general, higher *LEPR, NNAT* and *PER2* and lower *CA2* mRNA expression in HP compared to LP was observed in RNA-seq and validated by qRT-PCR, indicated lower adiposity in HP versus LP.

3.4.5 Fatty acids composition comparison

3.4.5.1 Comparison between Holstein and Hereford

3.4.5.1.1 Analysis of fatty acid profiles

The end product of biohydrogenation are SFAs, predominantly palmitic acid and stearic acid (Buccioni et al., 2012). Consistent with this, these FAs had the highest relative abundance percentages in AT from both Holsteins and Herefords, compared to other SFAs (Figure 3.19). The ratio of stearic over palmitic acid was slightly higher in Holsteins at 1:1.38, compared to 1:1.13 in Herefords. Comparison of FA composition in subcutaneous AT of Jersey and Limousin cattle, a dairy versus beef breed by Malau-Aduli and colleagues (1997) revealed a lower degree of SFAs (47.5 versus 52.9%, $p< 0.01$), but higher MUFAs (49.3 versus 45.3%, $p < 0.01$) and PUFAs (3.2 versus 1.8%, $p < 0.05$) in AT of Jersey compared to Limousin cattle. This agreed with results of the current study, in which the Holsteins had a higher level of SFAs, but lower MUFAs composition than Herefords. By contrast, PUFA was lower in Holstein compared to Hereford, but the difference was relatively low, at only 0.26%. However, the differences between categories of FAs in Holsteins and Herefords were statistically insignificant using multiple t-test.

Pentadecanoic acid (PA) is a FA with an odd number of carbon molecules on the hydrocarbon chain, thus termed odd chain fatty acids (OCFA). Research interest on PA was raised due to its potential function as biomarker for milk and dairy product consumption in people, through measurement of its relative content in AT or serum samples (Smedman et al., 1999, Brevik et al., 2005).

De novo synthesis of OCFA was from condensation of malonyl-CoA with propionyl-CoA as the primer, instead of acetyl-CoA that typically resulted in palmitic acid (C16:0) as the dominant end product (Smith, 1994, Vlaeminck et al., 2006). An alternative pathway for OCFA synthesis was proposed by Emmaneul (1978), whereby a carbon molecule was removed from palmitic acid through α-oxidation pathway. The PA in milk fat was proposed to have come from absorption of biohydrogenation end product via the duodenum, as well as *de novo* synthesis that occurred in the mammary gland (Vlaeminck et al., 2006).

The relative abundance of PA was found to be significantly lower in Holsteins compared to Herefords (Figure 3.21). As PA was reported to be a substantial component of milk fat, the lower PA content in AT of Holsteins may indicate that there was less uptake into adipocytes, or it was mobilised from AT for milk production. Vlaeminck et al. (2006) showed that ratio of PA to margaric acid (C17:0) was 2:1 in milk, while the ratio in AT was 1:3 (0.40% : 1.20%). In the current study, the ratio was lower in Herefords at about 1:2 (0.72%:1.62%) compared to Holsteins 1:3 (0.40% : 1.20%, Figure 3.20). Moreover, research demonstrated a consistent inverse serum concentration of margaric acid to the amount of milk fat intake (Brevik et al., 2005). This could also suggest higher utilisation of C15:0 from AT of dairy cows to be incorporated into milk fat.

Oleic acid (C18:1n9t) was the most abundant MUFA, exponentially higher compared to other MUFAs that were detected to be below 5% (Figure 3.20). Stearic acid was the most abundant SFA, and presumably its corresponding MUFAs of 18-carbon chain, C18:1n9c and C18:1n9t, would also be of the

highest concentrations (Figure 3.20). Linoleic acid (C18:2n6c) and linolenic acid (C18:3n3) were found to be significantly different between the breeds. As these are EFAs that could not be synthesised in the body, the difference in their compositions between the breeds is likely be due to differences in exogenous intake, or utilisation to meet the requirement of physiological processes. Milk fat contains ~ 2.3% PUFAs with n-6:n-3 ratio about 2 : 3 (Månsson, 2008), if some of these were obtained from AT or if adipocyte uptake was decreased due to the demand for milk production, this may explain the lower abundance of linoleic and linolenic acids in Holsteins compared to Herefords.

The PUFAs such as linoleic acid (LA), linolenic acid (LNA), EPA and DHA were previously observed to inhibit prostaglandin-F2α (PGF2α) synthesis, which could lead to maintenance of the CL and P_4 production, one of the major factors for establishment and maintenance of pregnancy in cows (Thatcher et al., 1997). Therefore, this appeared to be crucial to prevent embryonic loss during the early stage of pregnancy (Mattos et al., 2000). In addition, *in vitro* culture of bovine oocytes revealed that LNA improved bovine oocyte maturation and post-fertilisation development to the blastocyst stage (Leroy et al., 2005, Marei et al., 2009). If the higher levels of linoleic and linolenic acids in Herefords was able to promote survival of embryos in early pregnancy, this would give Herefords an advantage over Holsteins in terms of reproductive performances. However, as the levels of EPA and DHA were below 0.1% and eliminated from analysis, comparison of these FAs between Holsteins and Herefords were not feasible under the condition employed. Also, the composition and concentration of FAs in serum and follicular fluid were

reported to be comparable (Sinclair, 2010). However this correlation has yet to be examined between levels in AT and follicular fluid, thus FA composition of AT may not directly reflect the level of FAs in follicular fluid. However, higher levels of PUFAs in AT could potentially indicate greater availability to be released and utilised by other organs.

3.4.5.1.2 Hierarchical clustering analysis

Hierarchical clustering analysis failed to robustly and reproducibly distinguish breeds on the basis of FA profiles (Figure 3.21). Results suggested that FA profiles of these animals may not be explicitly different in general, although content of individual FA may show a greater disparity between breeds. It may be of interest to explore this further in order to further understand the intricacies of FA content in AT in relation to fat metabolism and reproduction.

3.4.5.2 Comparison between LP and HP

3.4.5.2.1 Analysis of fatty acid profiles

In parallel to FA composition of peri-renal AT of Holsteins and Herefords, SFAs were the main component of FAs in LP and HP (Figure 3.22), which is expected for tissues of ruminants (Lourenco et al., 2010). MUFAs were the next highest FAs, and PUFAs only made up a relatively small quantity of total FAs. As these animals were within the same age group and under similar management system, any change in composition was likely attributable to dietary source. However, unlike the composition breakdown between breeds,

FA profiles of AT from LP and HP were similar, and the differences between categories of FAs were not statistically significant (multiple t-test). As described previously, depletion of *Per2* led to an elevation of SFAs and MUFAs in mice (Grimaldi et al., 2010). However, in the current study, the amount of SFAs and MUFAs was not significantly higher in LP compared to HP, although *PER2* mRNA expression was reduced in LP. Furthermore, gene associated with elongation and lipogenesis, such as *ELOVL5*, *ELOVL6*, *FASN*, and *SCD*, did not display significant DE between diets.

Palmitic and stearic acids were the predominant FAs, and the ratio of both SFAs were identical at 1.14 for both LP and HP (Figure 3.24). Ratio of stearic to palmitic acid of LP and HP was observed to be similar to that of Herefords (1.13, Figure 3.23), another beef breed as AAX, while it was slightly higher in Holstein cattle, a dairy breed (1.38). Therefore, it is possible that FA uptake and utilisation may differ between dairy and beef breeds, although further work is required to determine the significance of this.

Oleic acid (C18:1n9c) was the most abundant MUFA, most likely due to desaturation of stearic acid, and perhaps also as a result of elongation then desaturation from palmitic acid. The LA had the highest percentage among PUFAs detected, and its composition was the only one found to be statistically different between LP and HP (FDR < 0.05). As LA concentration is dependent on exogenous sources, its higher level in HP may be due to higher percentage of grain based materials used compared to LP. However, this would require further confirmation as analysis of FA content in feedstuff and manipulation of FA concentration in tissue was beyond the scope of this study.

3.4.5.2.2 Hierarchical clustering analysis

Hierarchical clustering revealed that formation of clusters was driven by carcass conformation and fat class of the carcass, rather than dietary protein levels in the diet. It was also observed that conformation score was correlated inversely with fat class. This suggest that the level of FAs was associated with the ratio between muscle and fat in these cattle. Although dietary protein levels have been shown to influence adiposity in humans and rodents (Parker et al., 2002, Westerterp-Plantenga, 2003, Stepien et al., 2011), the differences between the two experimental groups may be too subtle to be detected at time of slaughter.

3.5 Conclusion

Expression of genes between AT of Holsteins versus Herefords, and LP versus HP in AAX cattle showed significant DE of genes involved in adipogenesis and lipogenesis between treatment groups. Future work should address whether the observed and validated changes in mRNA expression are reflected in protein expression of genes of interest. One challenge to this further work is the availability of appropriate antibody reagents for western blotting and ELISA analyses. Such analyses would be beneficial to advance understanding of physiological processes occurring in AT, and the effects AT has on other tissues and bodily functions of the animal as a whole.

FA profiles failed to robustly differentiate between AT of different breeds (Figure 3.21), and of AT following the low and high protein diet trial (Figures 3.24 and 3.25). However, comparison of individual FAs between Holsteins and Herefords revealed disparities in a number of these FAs that may have resulted from decreased uptake or increased mobilisation from AT to be incorporated in milk production. As outlined above, in the case of the protein diet trial, this may be attributable to the small differences between dietary protein levels tested or duration of treatment. Future studies should determine the digestibility and proportion of protein categories in both diets, as this study was limited to the profiling of AT. Future studies on the FA profiles of other biologically relevant tissues including milk and muscle are warranted. Significant differences detected between FA compositions were mainly of EFAs, and their concentration is dependent on exogenous sources as these FAs cannot be biosynthesised.

Chapter 4 Molecular Characterisation of Adipose Tissue of Large White and Meishan Pigs

4.1 Introduction

4.1.1 Origin of domestic pigs

Pigs are one of the nine existing wild and domesticated species in the genus Sus, within the Suidae family belonging to the Artiodactyla order (Porter, 1993b). The primary focus of this study was on domestic pigs (*Sus scrofa domesticus*), which are believed to be descendants of the Eurasian wild boar (*Sus scrofa*) based on zooarchaeological findings (Epstein and Bichard, 1984). Giuffra and colleagues (2000) utilised mitochondrial DNA (mtDNA) sequences and nuclear genes from wild and domestic pigs from Asia and Europe to confirm that both European domestic pigs and Chinese Meishan (MS) pigs are closely related to existing species of the Eurasian wild boar. Using inter-populational distances for mtDNA cytochrome B (ctB) gene sequences, they estimated that the ancestors of European and Chinese MS domestic pigs diverged from the Eurasian wild boar approximately 500,000 years ago (Giuffra et al., 2000).

On the other hand, studies of ancient and modern pig DNA sequences from numerous sites worldwide has identified multiple centres of pig domestication across Europe, West Asia, and East Asia (Giuffra et al., 2000, Larson et al., 2005, Larson et al., 2010). Furthermore, genetic data from these studies indicates that the European wild boar was the principal source of modern European domestic pigs, and these animals are separated from wild boar lineages from Asia that gave rise to the modern Asian domestic pig breeds

(Larson et al., 2005, Larson et al., 2010). Analysis of mitochondrial, microsatellite and Y-chromosome polymorphisms of pigs and wild boar from Europe, Asia, Africa and America further confirmed that European and Asia pigs are genetically separated, and microsatellite markers comparison between the European and Chinese modern domestic pig breeds showed a high heterozygosity index of 0.86 out of 1.0 (Megens et al., 2008, Ramirez et al., 2009).

4.1.2 Pig as food supply and its economic contributions

Pigs are important food source for people because of the pigs' ability to convert low quality plant and animal products and by-products to high quality protein. Traditionally, pigs were scavengers, and have previously been used as a convenient and productive mean of utilising food waste. In some parts of the world, pigs continue to be used in this way (Pond, 1991). Pork is the most widely consumed meat in the world, accounting for over 36% of the world meat intake, and 37% of world livestock production (FAO, 2014, Chemnitz, 2014). Similarly, consumption of pork, including bacon, ham and sausages, accounted for over 40% of total meat consumption in UK, the highest amount compared to beef, mutton, lamb and poultry (DEFRA, 2014). Furthermore, pork is an important economic commodity in UK. From January to November in 2014 alone, 244,301 tonnes of pig products, including offal, was exported from UK, and this value has been steadily increased from 217,126 tonnes in 2012 and 240,404 tonnes in 2013 (BPEX, 2015).

4.1.3 Development of pig breeds

A pig breed is defined by characteristics selected for over time, such as skin and coat colour, body size, ear carriage, fertility, mothering instinct and hardiness. Breeds are recognised by an organised breed association or by a government agency (Pond and Mersmann, 2001). As mentioned before, the divergence of the ancestors for European and Chinese MS domestic pigs occurred approximately 500,000 years ago. Adaptation to a wide range of local climates and available natural resources eventually resulted in a wider range of regional types, that were further enhanced over years of selective breeding (Porter, 1993a).

Typically, the formation of pig breeds in Britain during the late 18th and 19th century was accomplished through intense inbreeding of more productive pigs, until a distinctive type was established (Epstein and Bichard, 1984). As this distinctive type increased in popularity and more farmers began to keep and breed these animals, a new breed was successfully created. A breed society was then founded to maintain a central herdbook and oversee the pedigree of the breed (Epstein and Bichard, 1984). As the human population moved into new territories, pigs were brought into regions that lacked native pig populations, such as the United States of America (Epstein and Bichard, 1984). Imported breeds were then further developed to suit the new climatic conditions and farming systems. Improvement of productivity in the existing local population also prompted the importation of foreign breeds for crossbreeding purposes. This resulted in the development of new breeds of

pigs, and this approach is still being practiced in the industry today (Epstein and Bichard, 1984).

The European Large White (LW) and MS are the breeds selected for this study as the LW is the most popular breed for pork production in UK (BPA, 2015), while the MS are known for their high prolificacy and adiposity (Bidanel et al., 1990, White et al., 1996). The LW was developed in Yorkshire during the 19th century from local white breeds. During the 20th century, this breed grew in popularity and was one of the breeds, along with Landrace and Welsh, recommended to form the basis of the UK pig farming industry in the 1955 Howitt Report: Development of Pig Production in the United Kingdom (BPA, 2015). Meanwhile, MS pigs originated from the Mid Subtropic Belt area, a narrow region between north and central China, in the lower Changjiang River Basin and Southeast Coast lakes and valleys in China. These animals are considered as Taihu pigs, named after the Taihu Lake in their region of origin (Johnson et al., 1995).

4.1.4 Reproduction and influencing factors of fertility in female pigs

In general, gilts reach sexual maturity at 150 to 200 days, although some Asian and miniature breeds can reach puberty earlier, between 90 and 120 days (Pond and Mersmann, 2001). In addition to genetics, nutrition, social stress and environmental factors may also influence age at puberty in pigs (Pond and Mersmann, 2001). Generally, gilts are mated at their second or third oestrous cycle after puberty in order to take advantage of the increase in ovulation rate

between the pubertal and subsequent oestrous cycles (Kirkwood and Aherne, 1985).

After farrowing, pigs experience a lactational anoestrus period (Soede et al., 2011, Schwarz et al., 2008). Follicular development is inhibited during lactation, and weaning of the piglets allows recruitment and selection of follicles that will undergo preovulatory maturation and ovulation. Lactation inhibits gonadotropin-releasing hormone (GnRH) secretion, and in turn luteinizing hormone (LH) secretion, through neuroendocrine stimuli that are induced by suckling (Quesnel, 2009). Pituitary response to GnRH and the sensitivity of the hypothalamus-pituitary unit to estrogen positive feedback are also reduced (Quesnel, 2009). The impact of lactation on the reproductive axis is further complicated by the physiological and metabolic adaptations developed for milk production and are dependent on nutrient intake, nutrient needs, and body reserves. A strongly catabolic state during lactation amplifies the inhibition of LH secretion, thereby inducing a delay in weaning to oestrus intervals (Quesnel, 2009).

4.1.4.1 Nutritional influence on sow fertility

Adoption of artificial insemination in commercial pig production allows producers to substantially increase their sow-to-boar ratio, and be able to use more expensive, higher quality boars in their breeding programmes (Plain and Lawrence, 2003). Thus, prolificacy of sows became even more important to ensure good productivity. Prolificacy has been defined as the number of viable piglets produced per year or per breeding lifetime. A number of factors

influence prolificacy of sows, including the age at first successful mating, ovulation rate and embryo survival at each mating, number of live born, viable pigs and the sow's ability to be successfully remated at regular intervals (Aherne and Kirkwood, 1985).

A major consideration when choosing when to breed gilts is the length of their productive life. Delaying breeding until the gilt has achieved its adult weight and sufficient amount of back fat maximise the length of its productive life (Kirkwood and Thacker, 1992). Modern sows are leaner and younger at time of mating due to selection for leaner and high productivity compared to sows 30 to 40 years ago (Aherne and Williams, 1992). Therefore, feeding strategies should aim to minimize fluctuations in live weight and fat reserves, as well as maintenance of body condition throughout the animal's reproductive life to ensure long-term performance (Aherne and Williams, 1992).

A way to improve fertility of gilts through feeding is by providing extra food to the animals before the onset of second or third oestrus. This increases ovulation rate of feed restricted gilts to the levels achieved by gilts fed *ad libitum* (Aherne and Williams, 1992). During gestation, maintenance energy represents 75 to 85% of total energy requirements. Feeding an extra 1kg of feed per sow per day for the last 10 days of gestation prevents loss of sow back fat, at the same time increases piglet birth weight. Wherever possible, sows should be fed *ad libitum* from the day after farrowing until weaning. (Aherne and Williams, 1992, Vinsky et al., 2006, Hoving et al., 2012). As anticipated, reduced feed intake by lactating sows, results in excessive weight and condition loss (Aherne and Williams, 1992, Vinsky et al., 2006, Hoving et

al., 2012). This in turn causes extended remating intervals, a lower percentage of sows returning to oestrus within 10 days of weaning, reduced pregnancy rates, and reduced embryo survival (Aherne and Williams, 1992, Vinsky et al., 2006, Hoving et al., 2012). Sows with a delayed return to oestrus also have a lower pregnancy rate, and subsequent litters from these sows are smaller. A high level of feeding in the post-weaning period to replace the loss of weight or body condition during lactation, has been shown to improve embryo survival (Aherne and Kirkwood, 1985, Aherne and Williams, 1992).

4.1.4.2 Adipose influence on pig reproduction

Study results suggest that body weight is a more consistent determinant factor of the occurrence of the first oestrus in gilts than age of the animal (Dickerson et al., 1964, Friend et al., 1981). Severely undernourished prepubertal gilts with depressed growth rates were reported to have a delayed onset of puberty (Friend et al., 1981). Development of primordial follicles in the ovaries was not inhibited in gilts under prolonged undernutrition, however oestrus and ovulation did not occur. Upon realimentation, ovulation occured, but only after the gilts approached the "normal" ovulatory weight (Dickerson et al., 1964). However, other studies have indicated that increased body weight due to improved energy intake did not accelerate onset of puberty (Friend, 1977, Kirkwood and Aherne, 1985). Research in which feed is severely restricted for extended periods to deplete body reserves, which would impede physiological processes such as puberty onset, ovulation and lactation, is no longer feasible due to ethical issues related to such an intervention (Carstairs et al., 1980,

Kirkwood and Aherne, 1985). Furthermore, such poor body condition is unlikely to occur in standard production systems (Kirkwood and Aherne, 1985, Carstairs et al., 1980). However, it has been possible to determine the effect of low body fat composition on fertility in prepubertal girls with poor nutrition, anorexic women and female athletes undergoing strenuous training (Frisch and McArthur, 1974, Frisch, 1987). Therefore, body composition combined with age, may be a more appropriate indicators of reproductive maturity than either age or weight alone, as fat deposition had been reported to increase with age (Mitchell et al., 2001). In LW x Wessex neonatal piglets (day 1 to 30), the chemical composition comparison between protein and fat revealed that until 15 days of age, protein accretion was higher than fat, however percentage of fat overtakes protein from that point onwards. However, increment of protein and fat were parallel from day 15 till 30 (Manners and McCrea, 1963, Mitchell et al., 2001).

In general, leaner conventional pigs have higher growth rates compared to the fatter pigs. Assuming that adipose tissue (AT) developed initially by hyperplasia during the growth period and then later in life by hypertrophy and lipid deposition, the leaner conventional pigs would have more adipocytes than the fatter pigs during the growth period (Hood and Allen, 1977). Over time, however, fatter pigs would accumulate more adipocytes than the leaner animals as they would have an extended growth period compared to the lean pigs, and adiposity in the pigs would be due to cellular hypertrophy rather than cellular hyperplasia (Hauser et al., 1997). Also, the number of adipocytes per animal or per adipose depot is directly related to the true body size and weight of the fat-free carcass of the animal. Thus, the total number of adipocytes in

animals would likely be similar in lean and fat pigs (Hood and Allen, 1977), although the size of adipocytes would be enlarged in fat pigs (Hauser et al., 1997).

A comparison between MS, a breed with higher adiposity, and LW, a contemporary lean breed, demonstrated that sexual maturity was attained at an earlier age for MS gilts (Bolet et al., 1986, Bazer et al., 1988a, Bazer et al., 1988b). The MS gilts also recorded lower number of embryonic death, higher ovulation rate, higher number of live births, and higher number of survive neonates compared to LW gilts (Bolet et al., 1986, Bazer et al., 1988a, Bazer et al., 1988b). Numerous studies investigated the basis of the high prolificacy of the MS sows, however the molecular mechanism behind this phenomena is yet to be fully understood (Bazer et al., 1988b, Bolet et al., 1986, Bazer et al., 1988a, Bidanel et al., 1990, Hunter et al., 1993, White et al., 1993b, Lee et al., 1995, Haley et al., 1995, White and Wheeler, 1995, White et al., 2000). Studies on hybrid sows (Polish LW × Polish Landrace, Pig Improvement Company (PIC), and Dalland hybrid) showed that animals with higher back fat thickness have a more favourable outcome in numbers of piglets stillborn, litter weight at weaning, the average piglet weight at weaning and placental weight compared to leaner pigs within the same breed (Maes et al., 2004, Beyga and Rekiel, 2010). Also, loss of back fat during lactation was observed to precede decreased reproductive proficiency in pigs (Maes et al., 2004). Although further study is required to uncover the various elements influencing the reproductive performance of these animals, it is plausible that higher adiposity of MS pigs may endow a reproductive advantage over the LWs.

4.1.4.3 Effects of leptin in pig reproduction

Adipokines are cytokines predominately or exclusively expressed by AT that circulate and affect target tissues. Leptin (LEP) is currently the most reported adipokine in relation to reproduction in pigs (Barb et al., 2001, Gregoraszczuk et al., 2003, Barb et al., 2005, Barb et al., 2008, Hausman and Barb, 2010, Astiz et al., 2013). Adipocytes is the predominant site of LEP production, this is evident by higher levels of its mRNA and protein detected in obese pigs with greater fat mass volume and body fat percentage (Ramsay et al., 1998, Spurlock et al., 1998). Also, *LEP* mRNA expression in AT was found to increase with age and adiposity (Barb et al., 2006). Conversely, fasting-induced down regulation of porcine *LEP* mRNA abundance in subcutaneous AT which correlated with decreased adiposity (Ramsay et al., 1998, Spurlock et al., 1998). This can be related to reports of lower *LEP* expression in feed restricted pigs, as a result of reduced insulin and increased non-esterified fatty acids (NEFAs) in blood circulation (Spurlock et al., 1998, Barb et al., 2001).

LEP was proposed to initiate and modulate oocyte maturation and follicular development in swine (Moreira et al., 2013). The expression of *LEP* and *LEPR* genes was detected in granulosa cells (GCs), theca interna (TIC) and theca externa (TEC) cells. When comparing the expression level of *LEP* and its receptor's mRNA during the luteal phase of the oestrus, significantly higher levels of *LEP* gene expression in porcine GCs were observed during the mid- and late-luteal phases of the cycle. In early pregnancy, *LEP* mRNA expression of GCs was higher than those in luteal phase of the oestrous cycle. And the expression of the *LEPR* gene in GCs and TECs increased during pregnancy

in comparison with the luteal phases of the cycle. These observations indicated that locally produced LEP plays a role in the regulation of porcine reproduction at the ovarian level and exerts a direct effect on porcine follicles. The differences in *LEPR* gene expression in porcine GCs and theca cells also suggested that sensitivity to LEP varies in the ovaries of pregnant and cycling pigs (Smolinska et al., 2013).

LEP and *LEPR* transcripts and proteins were detected in porcine luteal cells by quantitative reverse transcription PCR (qRT-PCR) and fluorescence immunocytochemistry (F-ICC). *In vitro* studies on luteal cells from porcine follicles revealed that a higher level of *LEP* mRNA expressions in the cells was observed in the presence LH, 17β-estradiol (E_2) and progesterone (P_4). Also, radioimmunoassay (RIA) exhibited increased LEP secretion when the luteal cells were treated with E_2 and P_4 (Siawrys and Smolinska, 2013). During follicular phase of the oestrous cycle, *LEP* mRNA level was increased in growing follicles, and was highest in newly formed corpora lutea. The changes in expression level closely paralleled the concentration of P_4 (Gregoraszczuk et al., 2007). In addition, effect of exogenous LEP on steroidogenesis in follicles recovered from ovaries during early, middle, and preovulatory stages of the follicular phase of the oestrous cycle was examined. It appeared that a synergistic actions existed between LEP and follicle stimulating hormone (FSH) on E_2 secretion in small and medium follicles, as well as LEP and LH on P_4 secretion by large follicles in pigs (Gregoraszczuk et al., 2003). All these results indicate involvement of *LEP* in reproductive physiology of the pigs.

However, excessive amount of LEP may impair reproductive performance of sows. Obese Ossabaw pigs induced by excess-calorie, high-fat, high-cholesterol, high-fructose diet, were shown to develop abnormal reproductive function, such as longer oestrous cycles, higher serum androstenedione, and higher luteal phase serum LH, compared with control pigs (Newell-Fugate et al., 2014). The Iberian pigs, a breed prone to obesity was reported to have poor reproductive prolificacy due to embryo losses in early pregnancy (Astiz et al., 2013). Luteal functionality during the oestrous cycle and early pregnancy of Iberian sows and Large White x Landrace (LWxL) females was compared. The secretion of P_4 from the corpora lutea of obese Iberian sows was always hampered when compared to LWxL sows, either during early oestrous cycle, late oestrous cycle or early pregnancy (Astiz et al., 2013). The differences in basal P_4 secretion remained after stimulation with LH. Also, P_4 secretion during early pregnancy of Iberian sows decreased with age as the obesity features continued (Astiz et al., 2013). In both studies, the obese Ossabaw and Iberian sows exhibited hyperleptinemia (Astiz et al., 2013, Newell-Fugate et al., 2014). Administration of supraphysiological concentrations of LEP on small- and medium-sized ovarian follicles collected from prepubertal and cycling pigs resulted in elevated P_4 and testosterone secretions. This could be an independent risk factor for cyst formation in both prepubertal and cycling pigs and hinder reproductive performance of these animals (Gregoraszczuk and Rak-Mardyła, 2013). On the other hand, LEP insensitivity, a common consequence of severe obesity, may be the cause of the depression of P_4 secretion in the Iberian pigs (Astiz et al., 2013, Gregoraszczuk and Rak-Mardyła, 2013).

4.1.5 Nutritional factors on pig body and pork composition

Consumer choice is one of the major driving forces influencing pig body and pork composition. In general, a majority of consumers prefer lean meat as it is considered better value for money, and fat is perceived to have detrimental effect on health (Ngapo et al., 2004, Dransfield et al., 2005, Fortomaris et al., 2006, Ngapo et al., 2007, Chen et al., 2010, Ngapo et al., 2010). The amount of fat in pork can be changed through various ways, including genetic selection, feed formulation, even housing and production system (Lebret, 2008). Genetic selection coupled with increased understanding of nutrition has led to improvements in the efficiency of animal production and in carcass composition and quality, with respect to carcass fatness and muscle yield (Dunshea et al., 2005). With the economic challenges related to high global grain prices, the goal of meat animal production is to partition as much of the nutrient intake as possible into skeletal muscle tissue growth. This is imperative to address the urgency of global food security in response to the increase of human population and to prevent the repeat of food price increase that occurred in 2008 (GFS, 2015).

The focus of the current study was to identify differentially expressed genes (DEG) between LW and MS AT, and relate these to potential feed strategies because modification of feed formulation would likely produce significant changes in a shorter period of time compared to genetic selection. Therefore, the following sections would touch on effects of some nutritional compounds on adiposity and adipogenesis, as AT had been described to influence

reproductive performances in previous sections of this chapter, and in Chapter 1 Section 1.2.

4.1.5.1 Feed energy content

The feeding regime is one of the most important components influencing growth rate and composition of carcass in meat animals, besides genotype, sex and environment. Average daily weight gain was found to increase linearly with metabolisable energy (ME) intake in growing pigs on *ad libitum* and restricted feeding. Daily protein and lipid deposition were also noticed to be higher in animals fed *ad libitum* compared to ones on restricted diet (Quiniou et al., 1995). Restricted feed allowance lower energy intake of pigs, thus strongly reduced growth rate, carcass fatness, and intramuscular fat (IMF) level (Lebret, 2008).

4.1.5.2 Protein

From the previous chapter, alteration of protein level in feed has resulted in differential gene expressions of AT in cattle that may affect the level of fat deposition. It appears that dietary protein can produce similar effect in pigs. While maintaining energy levels in the diet, alteration of dietary crude protein levels changes growth rate and fat deposition in pigs (Campbell et al., 1984). Increased levels of dietary protein resulted not only in faster growth and protein deposition, also in carcasses containing less fat and higher amount of lean meat. However, growth rate will increase until an optimal level of protein is

reached at 22%, thereafter the growth rate decline, and protein deposition also plateaued (Cooke et al., 1972). Conversely, the rate of fat deposition continued to decrease as the protein content of the diet increase (Cooke et al., 1972). Therefore, growth depression at high levels of protein intake may be attributed to the decreased rate of fat deposition, as well as increment of heat production and a reduction in the efficiency of energy utilization (Campbell et al., 1985).

4.1.5.3 Vitamins

4.1.5.3.1 Vitamin A

The effect of vitamin A on growth rate, feed intake, or carcass composition in pigs was not reported. Literature search only uncovered one report that showed pigs fed on a diet without supplemental vitamin A had higher percentages of longissimus intramuscular fat without having detrimental effects on growth performance (D'Souza et al., 2003). However, the feed used also had reduced protein:energy ratio, and higher protein level in diet was earlier reported to reduce fat deposition (Cooke et al., 1972, Campbell et al., 1984, Campbell et al., 1985). Therefore, this study could not conclusively address the role of vitamin A on carcass composition in pigs. However, numerous reports in humans and rodent illustrated anti-adipogenic effect of vitamin A (Decsi et al., 1997, Kumar and Scarpace, 1998, Strauss, 1999, Ribot et al., 2001, Mercader et al., 2006, de Souza Valente da Silva et al., 2007, Berry and Noy, 2009, Villaca Chaves et al., 2008). Further studies are therefore required to determine the function of vitamin A on fat deposition in pigs.

In vitro study using stromal-vascular cells harvested from porcine AT showed that all-*trans* retinoic acid (ATRA), biological active form of vitamin A, inhibits porcine preadipocyte differentiation, promotes activation of RA receptor (*RAR*), and downregulates mRNA expression of genes critical in adipogenesis pathway such as peroxisome proliferator-activated receptor γ (*PPARγ*), and sterol regulatory element binding protein 1 (*SREBP1*); (Rosen et al., 1999, Eberle et al., 2004, Brandebourg and Hu, 2005). Additionally, ATRA reduced expression of *LEP* mRNA and LEP secretion in both human explant AT and murine 3T3-L1 adipocytes (Hollung et al., 2004). Indeed, as will be outline later, RNA sequencing (RNA-seq) of porcine AT indicated differential expression (DE) of genes related to vitamin A metabolism that potentially could be utilised in feed formulation targeted at specific breed to achieve the desired carcass composition. Modification of nutritional components in feed is fairly easy to accomplish and very likely to achieve significant results in relative shorter period of time, compared to manipulation of gene expression and selective breeding exercise.

4.1.5.3.2 Vitamin D

Vitamin D has not been extensively studied in pigs. This may be due to the contrasting effects on adipogenesis between species and at different ages. Calcitriol or 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), the biological active form of vitamin D, inhibited *in vitro* proliferation and differentiation of porcine preadipocyte, yet stimulated the differentiation of porcine mesenchymal stem cells (MSCs) towards adipogenesis (Zhuang et al., 2007, Mahajan and Stahl,

2009). In mice, calcitriol and its receptor, vitamin D receptor (VDR), inhibited the formation of adipocytes in mouse 3T3-L1 cell line (Blumberg et al., 2006, Kong and Li, 2006), but promoted the differentiation of primary mouse preadipocytes by increasing *PPARγ* mRNA expression (Nimitphong et al., 2012). The VDRKO mice were observed to resist diet induced weight gain, and the expression level of uncoupling protein 1 (*UCP1*) is elevated in AT. Although expression of *PPARγ* or fatty acid synthase (*FASN*) was not affected in AT of VDRKO mice (Narvaez et al., 2009). Plasma concentration of 25-hydroxyvitamin D (25(OH)D), a prohormone hydroxylated from vitamin D_3 (cholecalciferol), was inversely correlated with weight, body mass index (BMI) and fat mass in humans, and more than half of obese women were reported to suffer from vitamin D deficiency compared to women with normal body weight (Vilarrasa et al., 2007, Jungert et al., 2012). Also, low level of vitamin D and ATRA has been observed to significantly inhibit *in vitro* LEP secretion from human omental AT (Menendez et al., 2001). In contrast, calcitriol induced differentiation of human preadipocytes, and promoted triglyceride accumulation in newly-differentiated adipocytes and primary cultures of mesenchymal progenitor cells derived from human subcutaneous AT (Nimitphong et al., 2012, Narvaez et al., 2013).

4.1.6 Comparison between Meishan and Large White

4.1.6.1 Comparison of physical attributes

The physical appearance of LW is lean, with an elongated body, white coat and skin, distinctive prick ears and straight snout. Adult sows can grow up to

260 – 300kg, whereas boars can weigh 350 – 380kg in live weight. LWs are fast growing, well known for their excellent feed conversion ratio, thus are well suited to the commercial market (RBST, 2011). In other European countries besides UK, as with the United States, LW are known as Yorkshire, while some classify Yorkshire as a direct descendant from LW (Porter, 1993a).

The MS pigs are slow growing and fat, and able to consume large amounts of roughage. Generally the coat colour of MS pigs are dark, and the wrinkled face and skin are distinctive characteristics of this breed. Compared to the LWs, MS pigs appear short and rotund. Sows can grow to be about 58cm high, 100cm around the chest, and approximately 60kg in live-weight , while the boars can grow up to over 160kg in weight (Borg et al., 1993, Johnson et al., 1995).

Age matched comparison of LW and MS pigs showed that the LWs have a higher weight gain, which could be due to the higher average daily feed intake compared to MS pigs. However feed conversion rate were observed to be similar in both breeds, thus the cause of lower feed intake in MS pigs could be the lower energy requirement for growth and maintenance due to its smaller size (White et al., 1993a). The higher feed intake could also lead to the greater growth rate of internal organs (White et al., 1995), which was reported to correlate with body weight gain during growth period (Doornenbal and Tong, 1981). Previous research showed that piglets with lower birth weight compared to their littermates (runts were identified as piglets with more than two standard deviations below the mean weight of the rest of the litter) tend to have a slower growth rate, lower feed intake, reduced prenatal myogenesis resulting in lesser

muscle fibres and lighter muscle, as well as higher fat deposition (Powell and Aberle, 1980, Wigmore and Stickland, 1983, Rehfeldt and Kuhn, 2006). This may be the underlying causes that could explain the difference of growth rate and body composition between Yorkshire and MS as the former has higher birth weight (White et al., 1993a, White et al., 1995).

Examination of the lean tissue from carcass cut data at 171 day of age indicated significant higher rate of protein deposition in LW than MS pigs (White et al., 1995). On the other hand, the percentage of body fat was higher in MS throughout the period of study (from 40 – 171 days of age), except at day 260 where both breeds were observed to have similar amount of body fat (White et al., 1995). From these results, it was concluded that MS pigs begin to accumulate fat at an earlier age, are less efficient in converting feed to lean weight, and have lower growth rate compared to LW.

4.1.6.2 Comparison of reproductive performances

The MS pigs have generated considerable interest for research primarily due to their high prolificacy and sexual maturity at an early age (Bidanel et al., 1990). In contrast to development of other internal organs, reproductive organs growth rate appear to be faster in MS compared to LW pigs. In the earlier stages, MS gilts have significantly heavier ovary and uterus weights, and attain puberty at 95 days of age, 105 days earlier than LW pigs (White et al., 1993b). However, by the time LW pigs attain puberty, the weights of the ovaries and uterus are similar between the two breeds (White et al., 1995).

This may suggest that LW pigs are larger, later-maturing pigs than MS pigs (Bidanel et al., 1990, White et al., 1995).

In addition, MS pigs are also reported to have longer oestrus period, shorter oestrous cycle, produce more ova, higher number of viable embryo during early pregnancy, approximately 10% lower conceptus mortality rate, consequently farrow 3 to 4 more pigs per litter than LW sows (Bolet et al., 1986, Bazer et al., 1988b, Hunter et al., 1993, Lee et al., 1995, Haley et al., 1995). On average, research found 1.86 mummified foetuses per litter from MS gilts as opposed to only 0.05 per LW litter, yet MS still produce a higher number of viable live births per litter. This may be due to the higher ovulation rate in MS pigs compared to LW pigs (White et al., 1993b).

Statistical analysis of farrowing records on 969 litters from over 500 sows of four genetic types, LW, MS, Laconie and Duroc x LW, showed that the five most important factors affecting piglets probability of stillbirth are: differences between piglet birth weight and the litter mean, individual birth weight, piglet sex, farrowing duration, and sow genetic type (Canario et al., 2006). Also, the probability of stillbirth was greater for lighter piglets, male piglets, and piglets from small or very large litters (Canario et al., 2006). Incidentally, occurrence of stillbirth increased with farrowing duration and with sow parity number. However, piglets born from MS sows were reported to have a lower risk of stillbirth and were little affected by the five factors mentioned above compared with the 3 other sow genetic types (Canario et al., 2006).

4.1.6.3 Functional and physiological differences between Meishan and Large White pigs in relation to reproduction

4.1.6.3.1 Ovary

The MS gilts had a higher ovulation rate than LWxL gilts (Ashworth et al., 1997). A comparison of ovulation rate from sows of 3rd and 4th parity to ones with 7th parity showed that a proportionately greater increase in ovulation rate occurs in MS after they have experienced pregnancy compared to European breeds (Hunter et al., 1993). Moreover, it was noticed that MS had a longer interval between onset of oestrus and ovulation compared to LW, being 49.0 and 34.3 hours respectively (Wilmut et al., 1992). Through administration of human chorionic gonadotropin (hCG), the time of oestrus onset to LH surge in MS gilts was advanced to be similar to that of European breeds. Although the ovulation rates did not differ between treated gilts with control animals, the number of live conceptuses and embryo survival rate declined drastically (Hunter and Picton, 1995). This can be explained by the observation that oocytes from Meishan pigs are in a more advanced stages of meiotic maturation compared to oocytes from Large White pigs at the time of ovulation (Faillace and Hunter, 1994). Therefore, it was concluded that the longer period between oestrus onset and ovulation is an important factor for the higher rate of embryo survival in the MS (Hunter and Picton, 1995).

4.1.6.3.2 Uterus

The uterus of farrowing MS and LW pigs are similar in size (Bazer et al., 1988a, Lee et al., 1995, Wilson et al., 1998), yet embryos and piglets born to MS sows are lighter than those from LW sows (White et al., 1993a, White et al., 1995), with smaller spaces between embryo sites (Wilson et al., 1998). In addition, embryonic development and conceptus growth was reported to be slower in MS pigs compared to LW pigs (Ashworth et al., 1990, Vallet et al., 1998, Biensen et al., 1999). Thus, limiting the size of the foetuses and decreasing the area of endometrial surface required per conceptus is a mechanism proposed for sustaining a higher number of foetuses in the MS pigs (Fenton et al., 1972).

4.1.6.3.3 Placenta

A comparison of placenta between MS and LW pigs indicated that there is a lack of placental growth during late gestation of MS conceptuses, and the vascular density of MS placentae and adjacent endometrium are more than double than that of LW from day 90 to 110 of gestation (Biensen et al., 1998, Wilson et al., 1998). By contrast, LW placental surface area doubled during late gestation, but vascular density remained constant (Biensen et al., 1998). Therefore, Biensen and colleagues (1998) suggested that the increased nutrient uptake from a limited surface area of contact between the placenta and endometrium is a key factor to support the larger litter size of MS pigs (Biensen et al., 1998). In addition, a smaller, yet more efficient placenta in MS, takes up less space in the uterus than conceptuses of Western breeds, without

compromising the viability of the foetuses. Therefore, the enhanced placental efficiency allow more MS conceptuses to colonize the uterus compared with Western pig breeds (Wilson et al., 1998).

4.1.6.3.4 Endocrinology

The LH surge occurs later in the MS in relative to the onset of behavioural oestrus than in LW pigs, this means that MS pigs ovulate later from the time of oestrus onset compared to LW pigs (Wise et al., 2001). As circulating E_2 concentrations are not significantly different between the breeds (Wise et al., 2001), MS pigs appear to be more sensitive to E_2 in terms of initiating a behavioural response, but not in positive feedback, as the interval from peak E_2 to the onset of the LH surge was similar in both breeds (Hunter et al., 1993).

The P_4 concentration during the early luteal phase tends to be lower in pregnant LWxL gilts than in pregnant MS gilts (Ashworth et al., 1994). Furthermore, the maximum P_4 concentration occurs later in LWxL gilts than in MS gilts whether they are in cycle or during the gestation period (Ashworth et al., 1994). In non-mated animals, the decrease in P_4 concentrations occurred earlier in MS gilts when the timing and magnitude of P_4 concentrations were analysed towards the end of the oestrous cycle. Thus, it was proposed that breed differences in the peripheral P_4 profile may be associated with reduced prenatal mortality in MS pigs (Ashworth et al., 1994).

As mentioned in Chapter 1 Section 1.2.1, AT has been reported to participate in the regulation of the hypothalamic-pituitary-gonadal (HPG) axis, however the exact role of adipose depots in reproduction remains poorly understood. It

was hypothesised that the higher percentage of body fat in the MS compared to that of the LW pigs may be an important factor in enhancing reproductive efficiency. To test this hypothesis, RNA-seq was used to analyse the transcriptomes of AT from MS and LW neonates (7-day old). From among the DEG, functional significance of the identified gene networks was examined for a better understanding of differences in reproductive performance of the MS and LW pigs.

4.2 Materials and methods

4.2.1 Animals and samples

Seven-day old MS and LW female piglets kept on the same commercial farm during the same period of time were euthanized by pentobarbital injection, and samples were collected from piglets of similar body weight, as to not include the runt within the litters. Subcutaneous AT was collected and samples were frozen in liquid nitrogen and subsequently stored at -80^{o}C until analysis. Two adipose samples from each breed were sequenced (Table 4.2), and transcriptomic results were compared. An outline of sample processing and RNA-seq data analysis workflow was identical to that illustrated in Figure 2.1 (Chapter 2). Experimental protocols and operative procedures were reviewed and approved by the Home Office in accordance with the Animals (Scientific Procedures) Act 1986 (Fainberg et al., 2012), with local ethical approval of the Research Directorate and the Ethics Committee at the School of Veterinary Medicine and Science.

4.2.2 RNA-sequencing and differential expression analysis

Protocols for preparation of samples for sequencing and downstream analysis were as described in Chapter 2. Trimmed sequences for the pig adipose samples were aligned to the *Sus scrofa* genome assembly produced by Swine Genome Sequencing Consortium (SGSC); (Sscrofa10.2.71, August 2011) in the Ensembl database, encoding 21,630 coding genes, 2,989 small non-coding genes, 135 long non-coding genes, and 568 pseudogenes.

4.2.3 Synthesis of cDNA and quantitative reverse transcription PCR

The validation of RNA-seq results was performed on 10 subcutaneous adipose samples collected from 7-day old female piglets (MS n = 4, LW n= 6). Reverse transcription (RT) of 1µg of total RNA was performed using the HiFlex protocol of the Qiagen miScript II Reverse Transcriptase Kit (Qiagen, Crawley West Sussex, UK), in order to synthesise cDNA which can be utilised to analysis miRNA from the samples. The TaqMan® Gene Expression Assays hydrolysis probesets (Applied Biosystem, Carlsbad, CA, USA) used are listed in Table 4.1.

The extracted RNA template was thawed on ice, while the 10x miScript Nucleics Mix, RNase-free water, and 5x miScript HiFlex Buffer were thawed at room temperature (15 – 25ºC). The miScript Reverse Transcriptase Mix is extremely heat sensitive, thus it was kept frozen in -20ºC freezer until just before the preparation of the RT master mix was to begin. The miScript Reverse Transcriptase Mix was kept on ice at all times outside of the freezer

and was returned to the freezer immediately after use. Once they were thawed, each solution was mixed by flicking the tubes, then centrifuged briefly (9,632g for 10 seconds at 4^{o}C) to collect residual liquid from the sides of the tubes, and stored on ice.

The RT master mix was prepared by adding 2µL of 5x miScript HiFlex Buffer, 1µL of 10x miScript Nucleics Mix, 1µL of miScript Reverse Transcriptase Mix, and 4µL of RNase-free water in a microcentrifuge tube. These were gently mixed by inversion and placed on ice. The amounts of reagents stated above was for RT of a single RNA sample. Therefore, in order to perform RT for multiple samples, the amount of reagents used was increased accordingly. A total of 8µL of RT master mix was pipetted into a new microcentrifuge tube, and 2µL of RNA template was added. The mixture was inverted gently, briefly centrifuged (9,632g for 10 seconds at 4^{o}C) and stored on ice. Samples were then placed in a water bath at 37^{o}C and incubated for 60 minutes. After incubation, cDNA was diluted with 190µL of RNase-free water and stored at -20^{o}C.

Table 4.1 List of Taqman® hydrolysis probesets used for qRT-PCR.

Gene	Taqman® hydrolysis probeset ID
ACTB	Bt03279174_m1
CRABP2	Ss04245556_m1
LEP	Ss03392404_m1
UCP2	Ss03392404_g1
VDR	Hs01045840

4.3 Results

4.3.1 Transcriptomic analysis

4.3.1.1 Statistics and read annotations

RNA-seq using the Illumina Hi-Seq platform produced paired sequence reads, and the number of raw reads obtained, as well as breakdown of read numbers after quality control (QC) and mapping processes are depicted in Table 4.2 and Figure 4.1. Three of the samples were sequenced twice (MS1, MS2 and LW1) to produce sufficient number of reads as specified in the original contract with Edinburgh Genomics, whereas LW2 was sequenced once. However, raw reads generated from both sequences were pooled and normalised, thus higher read numbers in MS1, MS2 and LW1 would not result in greater mRNA expression levels. The percentages of aligned and QC reads were similar in all samples (Figure 4.2). A total of 25,322 of genes in reference to Ensembl database were annotated from the mapped reads, and these were used for subsequent analyses.

Table 4.2. Breakdown of RNA-seq reads for adipose samples of MS and LW pigs. Number of raw reads generated from RNA-seq are shown as total reads. QC reads are sequence reads kept after QC, and the ones that did not pass the QC processes are the discarded reads. Sequence reads that were confidently mapped to the reference genome are presented as mapped reads, leaving the ones that were not mapped as unmapped reads.

ID	Breed	Age	Total reads	QC reads	Mapped reads	Unmapped reads	Discarded reads
MS1	MS	7 d	61,094,566 (100%)	59,263,516 (97.00%)	47,302,857 (77.43%)	11,960,659 (19.58%)	1,831,050 (2.99%)
MS2	MS	7 d	62,397,766 (100%)	60,622,960 (97.16%)	49,956,870 (80.06%)	10,666,090 (17.09%)	1,774,806 (2.85%)
LW1	LW	7 d	78,253,934 (100%)	76,056,478 (97.19%)	63,243,747 (80.82%)	12,812,731 (16.37%)	2,197,456 (2.81%)
LW2	LW	7 d	48,974,692 (100%)	48,050,514 (98.11%)	39,033,449 (79.70%)	9,017,065 (18.41%)	924,178 (1.89%)

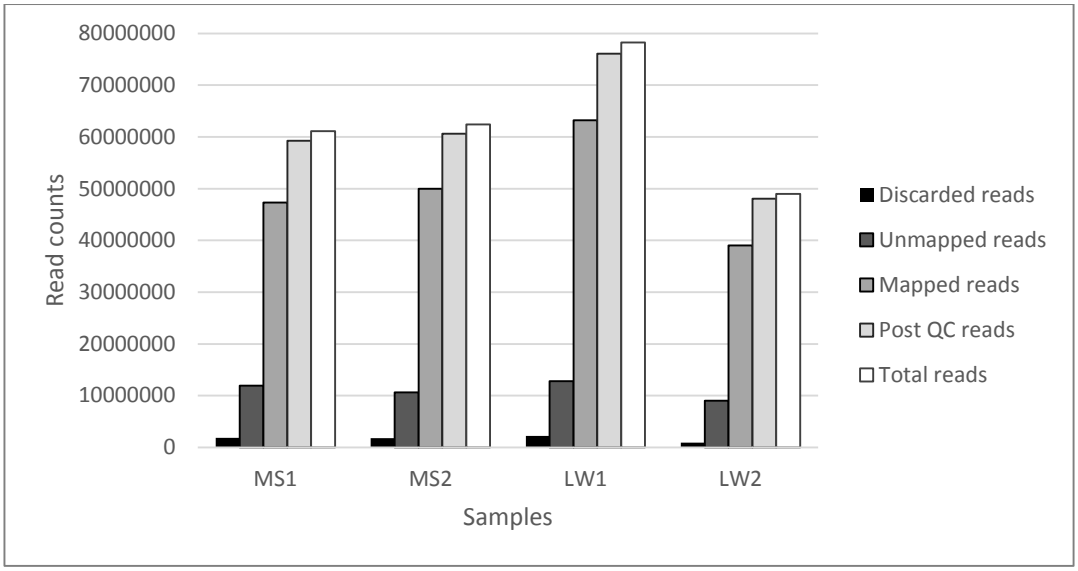


Figure 4.1. RNA-seq reads dispersions by count numbers for adipose samples of MS and LW pigs. Total reads refer to the number of raw reads generated from RNA-seq. Post QC reads are sequence reads that passed QC criteria, and the discarded reads were the ones that failed to pass QC. Mapped reads are sequence reads confidently mapped to reference genome, while the ones that were not mapped are presented as unmapped reads. LW2 had a lower number of read counts as it was sequenced once, whereas MS1, MS2, and LW1 were sequenced twice.

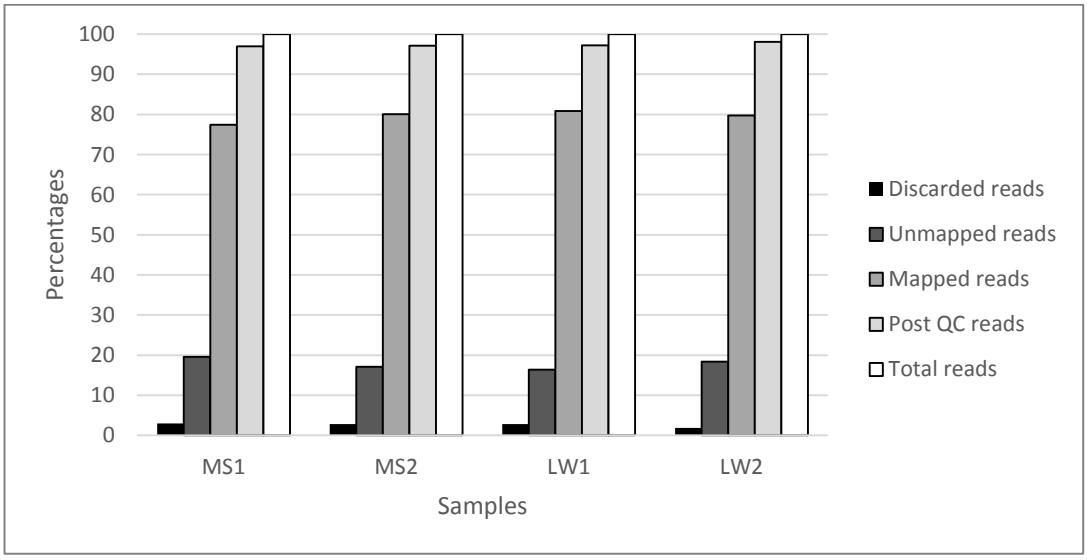


Figure 4.2. RNA-seq reads dispersions by percentages of count numbers for adipose samples of MS and LW pigs. This graph shows the breakdown of sequence reads in percentages corresponding to count numbers presented in Figure 4.1. Composition of LW2 appeared comparable with MS1, MS2, and LW1, although the count numbers was lower.

4.3.1.2 Differentially expressed genes

A total of 440 genes were found to be significantly differentially expressed between MS and LW through DE analysis using edgeR. Among these genes, expressions of 182 genes were lower, and 258 were elevated in MS compared to LW (Appendix 4). A multidimensional scaling (MDS) plot was used to show the similarity or dissimilarity of the samples. Each sample is represented as a point on the plot, and the distance between points corresponds to the similarities between them, calculated as biological coefficient of variation (BCV). Thus, similar samples are closer to one another, while dissimilar samples are further apart (Wickelmaier, 2003). Figure 4.3 shows replicates of MS and LW are clustered at parallel level in their respective breed, although MS replicates appeared to be closer to one another than the LW replicates. Smear plot and volcano plots shows the distribution of annotated genes (Figures 4.4 and 4.5). Filtering the gene lists for genes with a fold change (FC) $\geq$ 2, narrowed down the pool of genes of interest. A scatter graph comparing log FC over false discovery rate (FDR) of DEG shows higher number of genes at the lower FDR range on the y-axis, this illustrated significance of DEG (Figure 4.). FDR is the expected proportion of erroneous rejections among all rejections (Benjamini and Yekutieli, 2001), thus lower FDR value points toward a lower chance of significant values being false positives. Genes were selected for subsequent analysis by satisfying a FC $\geq$ 2 and FDR < 0.05.

The top 25 lower and higher expressed genes according to FC in MS compared to LW pigs are listed in Tables 4.3 and 4.4. Twelve of the top 25 genes with higher mRNA expression in LW were found to be related to

myofibril assembly, skeletal and cardiac muscle development, heart development, skeletal and heart muscle contraction (Table 4.3): *MYL3, TNNI1, TNNT1, MYH7, TNNC1, MYH6, CSRP3, CARP* (*ANKRD1*)*, MYH3, ACTC1,* and *TCAP* (Xie et al., 2003, Hundley et al., 2006, Landstrom et al., 2008, Posch et al., 2011, Foster et al., 2012, Pinto et al., 2012, Crocini et al., 2013, Francis et al., 2014, Greenway et al., 2014, Hoffmann et al., 2014, Nomura et al., 2015) according to information obtained from NCBI Reference Sequence Database, RefSeq (http://www.ncbi.nlm.nih.gov/refseq/). Functional activities of the top 25 genes with higher mRNA expression in MS (Table 4.4) are involved in various functions including transcriptional signalling, transmembrane transport of molecules. Genes are implicated in generalised tissue and organ development: *BMP7, WNT2* and *WNT4* (Chen et al., 2004, Katoh, 2003, Garcia-Castro et al., 2013), specifically related to meiosis: *SYCP2* (Offenberg et al., 1998), and lipid binding: *ALDH1A2* (Elizondo et al., 2000).

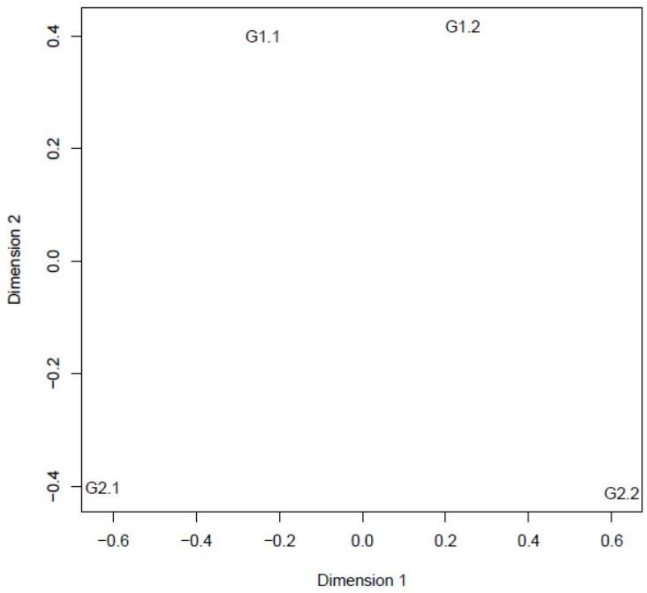


Figure 4.3. Multidimensional scaling plot on differential gene expression of AT in MS versus LW. Replicates of MS and LW pigs were clustered at parallel level in their respective breed, this shows that samples between breeds were distinctively different. MS replicates appeared to cluster closer together than LW replicates. (G1.1 = MS1, G1.2 = MS2, G2.1 = LW1, G2.2 = LW2).

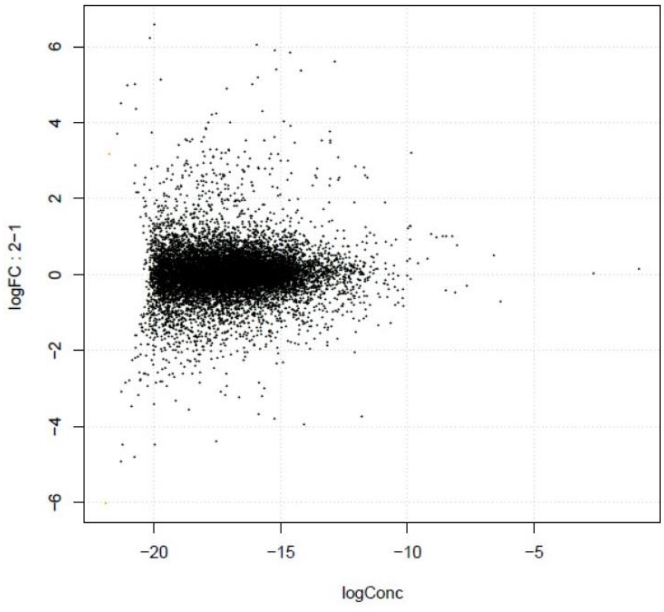


Figure 4.4. Smear plot comparing log FC and –log average gene expression counts on differential gene expression of AT in MS versus LW pigs. This plot shows distribution of annotated genes.

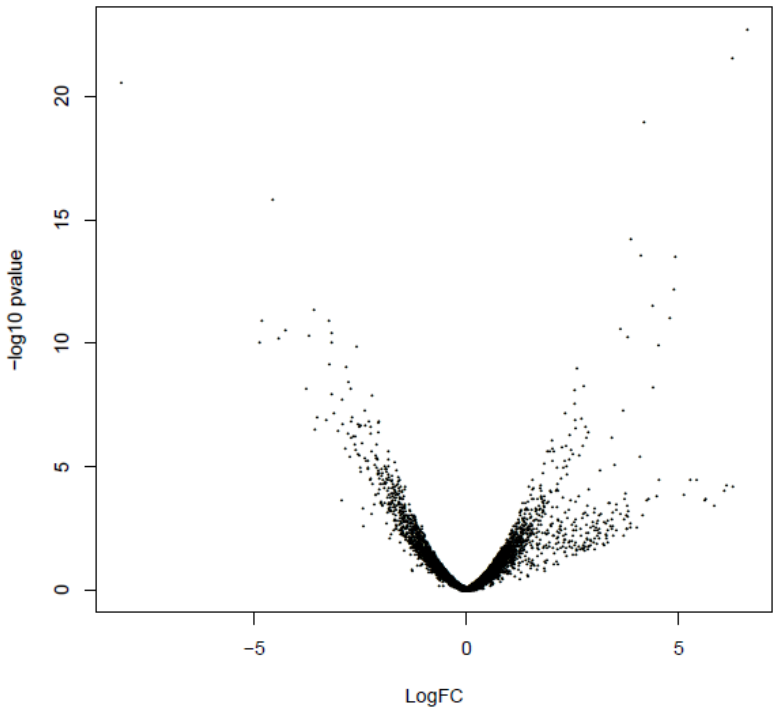


Figure 4.5. Volcano plot comparing -log10 p-value and log FC on differential gene expression of AT in MS versus LW pigs. Volcano plot with DE genes of interest with FC ≥ 2 in the upper left and right regions.

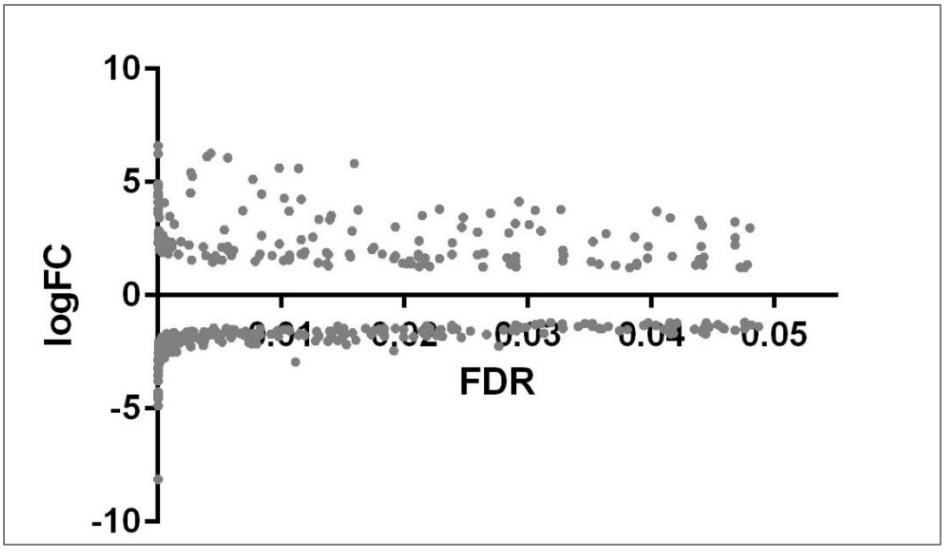


Figure 4.6. Differential gene distribution by log FC versus FDR on differential gene expression of AT in MS versus LW.

Table 4.3. Top 25 genes with lower mRNA expression in MS compared to LW pigs ordered by the amount of fold change. Twelve of the top 25 genes with higher mRNA expression in LW were found to be related to myofibril assembly, skeletal and cardiac muscle development, heart development, skeletal and heart muscle contraction (*MYL3, TNNI1, TNNT1, MYH7, TNNC1, MLC2V, MYH6, CSRP3, CARP, MYH3, ACTC1, and TCAP*).

Ensembl ID	Log FC	Official symbol	Gene description
ENSSSCG00000016516	6.615254	*ATP6V0A4*	ATPase, H+ transporting, lysosomal V0 subunit a4
ENSSSCG00000011325	6.270246	*MYL3*	Myosin, light chain 3 (alkali; ventricular, skeletal, slow)
ENSSSCG00000010007	6.259869	*MTFP1*	Mitochondrial fission process 1
ENSSSCG00000024061	6.129094	*TNNI1*	Troponin I type 1 (skeletal, slow)
ENSSSCG00000025353	6.071799	*TNNT1*	Sus scrofa troponin T type 1 (skeletal, slow)
ENSSSCG00000002029	5.833332	*MYH7*	Myosin, heavy chain 7, cardiac muscle, β
ENSSSCG00000011441	5.632467	*TNNC1*	Sus scrofa troponin C type 1 (slow)
ENSSSCG00000009830	5.610529	*MLC2V*	Sus scrofa myosin light chain 2V
ENSSSCG00000030999	5.422136	*MYH6*	Myosin, heavy chain 6, cardiac muscle α,
ENSSSCG00000013354	5.265918	*CSRP3*	Cysteine and glycine-rich protein 3 (cardiac LIM protein)
ENSSSCG00000010461	5.119656	*CARP*	Sus scrofa cardiac ankyrin repeat protein
ENSSSCG00000007505	4.881153	*CTCFL*	Sus scrofa CCCTC-binding factor (zinc finger protein)-like
ENSSSCG00000026162	4.792662	*LOC100628155*	Peptidyl-prolyl cis-trans isomerase
ENSSSCG00000018007	4.528666	*MYH3*	Myosin, heavy chain 3, skeletal muscle, embryonic
ENSSSCG00000010814	4.519739	*ESRRG*	estrogen-related receptor gamma
ENSSSCG00000021071	4.478103	*HSPB7*	heat shock 27kDa protein family, member 7 (cardiovascular)
ENSSSCG00000022737	4.389894	*MYD88*	Uncharacterized protein
ENSSSCG00000021375	4.379373	*HS6ST2*	Heparan sulfate 6-O-sulfotransferase 2
ENSSSCG00000004803	4.287977	*ACTC1*	Sus scrofa actin, α , cardiac muscle 1
ENSSSCG00000010303	4.24945	*SYNPO2L*	Synaptopodin 2-like
ENSSSCG00000024919	4.176521	*RDH16*	Retinol dehydrogenase 16 (all-trans)
ENSSSCG00000017500	4.147833	*TCAP*	Sus scrofa titin-cap (telethonin)
ENSSSCG00000015617	4.104784	*G0S2*	G0/G1switch 2
ENSSSCG00000024676	4.087545	*SRPK3*	SRSF protein kinase 3
ENSSSCG00000015835	3.803121	*DUSP26*	Dual specificity phosphatase 26 (putative)

Table 4.4. Top 25 genes with higher mRNA expression in MS compared to LW pigs ordered by the amount of fold change. Functional activities of top 25 genes with higher mRNA expression in MS are involved in various functions including transcriptional signalling, transmembrane transport of molecules. Genes are implicated in generalised tissue and organ development (*BMP7, WNT2*, and *WNT4*), specifically related to reproductive system development (*SYCP2*) and lipid binding (*ALDH1A2*).

Ensembl ID	Log FC	Official symbol	Gene description
ENSSSCG00000028674	4.55966	*CR2*	Complement component (3d/Epstein Barr virus) receptor 2
ENSSSCG00000026386	4.26379	*SLC30A2*	Solute carrier family 30 (zinc transporter), member 2
ENSSSCG00000001570	3.77443	*PI16*	Peptidase inhibitor 16
ENSSSCG00000015663	3.71187	*C4BPA*	Sus scrofa complement component 4 binding protein, α
ENSSSCG00000023591	3.5919	*GPR111*	G protein-coupled receptor 111
ENSSSCG00000002368	3.52004	*LTBP2*	Latent transforming growth factor beta binding protein 2
ENSSSCG00000025578	3.30958	*ALDH1A2*	Aldehyde dehydrogenase 1 family, member A2
ENSSSCG00000005638	3.22515	*LCN2*	Sus scrofa lipocalin 2
ENSSSCG00000007501	3.18143	*BMP7*	Bone morphogenetic protein 7
ENSSSCG00000013556	3.18044	*EMR1*	Egf-like module containing, mucin-like, hormone receptor-like 1
ENSSSCG00000025698	3.11933	*SERPINE1*	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
ENSSSCG00000030655	3.03108	*MAMDC2*	MAM domain containing 2
ENSSSCG00000021222	2.93687	*MCT7*	Sus scrofa tryptase
ENSSSCG00000011683	2.92698	*PCOLCE2*	Procollagen C-endopeptidase enhancer 2
ENSSSCG00000009332	2.91707	*TEX26*	Testis expressed 26
ENSSSCG00000003414	2.86092	*ANGPTL7*	Sus scrofa angiopoietin-like 7
ENSSSCG00000016628	2.83448	*WNT2*	Wingless-type MMTV integration site family member 2
ENSSSCG00000016129	2.79065	*GPR1*	G protein-coupled receptor 1
ENSSSCG00000006172	2.78196	*PI15*	peptidase inhibitor 15
ENSSSCG00000008737	2.72805	*C1QTNF7*	Sus scrofa C1q and tumour necrosis factor related protein 7
ENSSSCG00000016684	2.7225	*SCRN1*	Secernin 1
ENSSSCG00000003521	2.71442	*WNT4*	Sus scrofa wingless-type MMTV integration site family, member 4
ENSSSCG00000007529	2.70044	*SYCP2*	Synaptonemal complex protein 2
ENSSSCG00000000252	2.66667	*KRT8*	Keratin 8
ENSSSCG00000011472	2.63083	*DNASE1L3*	Deoxyribonuclease I-like 3

4.3.1.3 Hierarchical cluster analysis and gene ontology annotation

To visualise global gene expression across all the samples, a heat map was generated using Cluster 3.0 (bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and Java Treeview (jtreeview.sourceforge.net); (Figure 4.7). From the 25,322 genes that satisfied the initial QC criteria, these were filtered down to 1,152 genes where all samples have read counts $\geq$ 50, and FC $\geq$ 2. Samples from the same breed clustered together as anticipated, as shown by the dendrogram. This confirmed that the samples within the same breed more closely resembled each other than to the other breed. The heat map shows genes differentially expressed between breeds, where red represents genes with higher mRNA expression, and green represents the ones with lower expression.

Gene ontology (GO) term enrichment analysis using WebGestalt on lower expressed genes from MS pigs compared to LW pigs reciprocated results of top 25 genes listed according to FC (Table 4.3). Enriched categories, shown in red, are related to muscle development and functions as illustrated in the Directed Acyclic Graphs (DAGs); (Figure 4.8). Meanwhile GO terms of genes with higher mRNA expression in MS pigs compared with LW pigs demonstrated enrichment for extracellular spaces, wound healing and biological processes related to lipid (Figure 4.9).

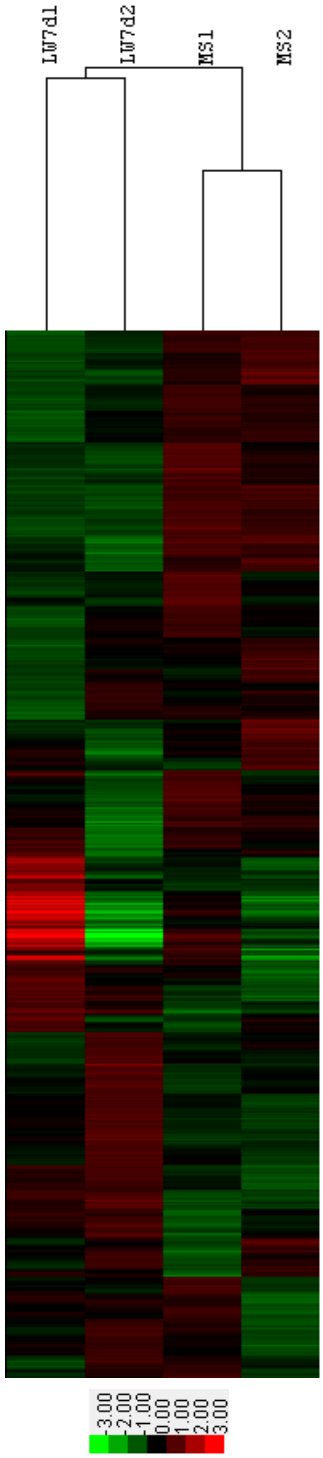


Figure 4.7. Hierarchical clustering of global gene expression between subcutaneous AT of MS and LW pigs. Heat map shows genes differentially expressed between breeds, where red represent higher mRNA expression, and green represents lower expression. From the total of 25,322 genes, the number of genes were filtered down to 1,152, where all samples have $\geq$ 50 read counts, and FC $\geq$ 2. Clustering of samples within each breed denoted by the dendrogram confirmed that the samples more closely resembled each other, compared to the other breed. MS n = 2, LW n = 2.

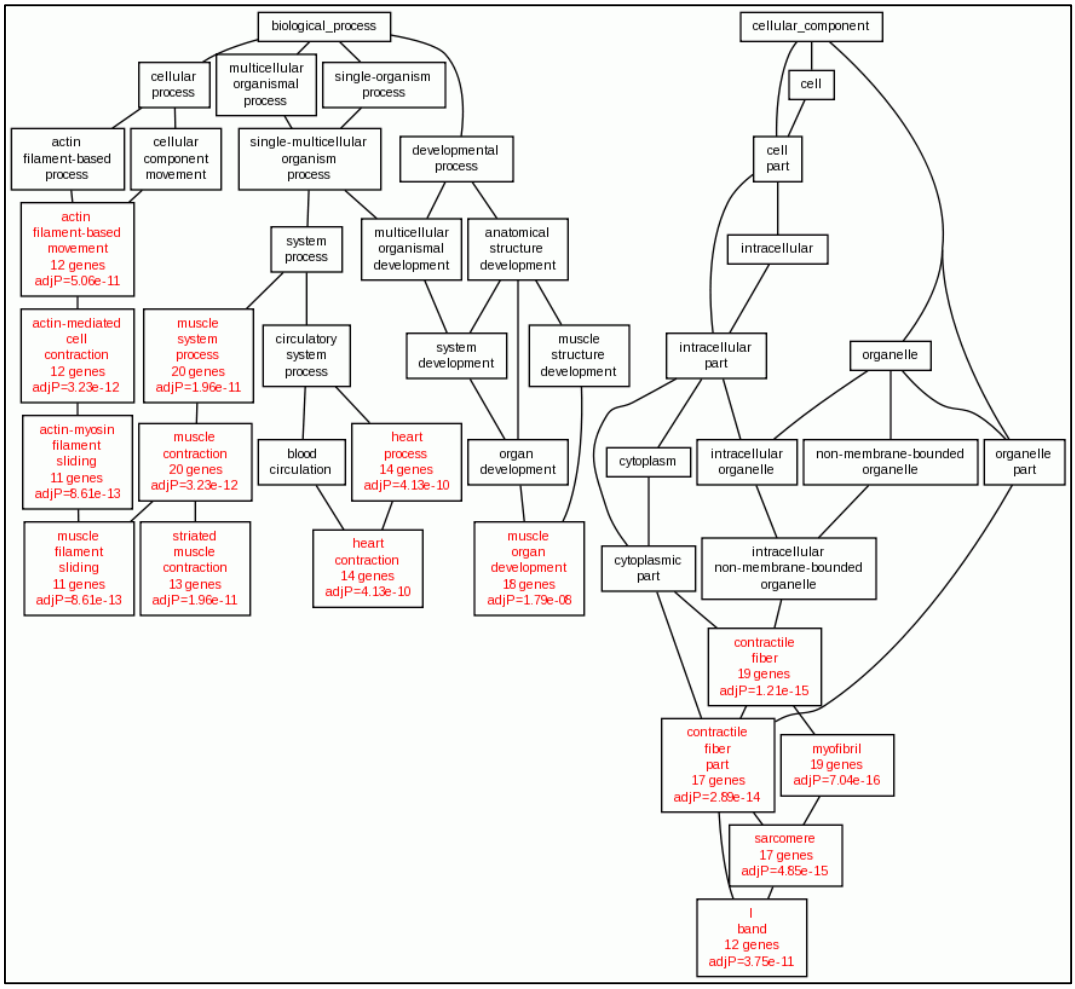


Figure 4.8. WebGestalt Directed Acyclic Graphs (DAGs) showing enriched GO categories under Biological Process, Molecular Function, and Cellular Component of genes with lower mRNA expression for MS compared to LW pigs by fold change. GO term enrichments are shown in red, and terms were related to muscle myofibrils, and the heart.

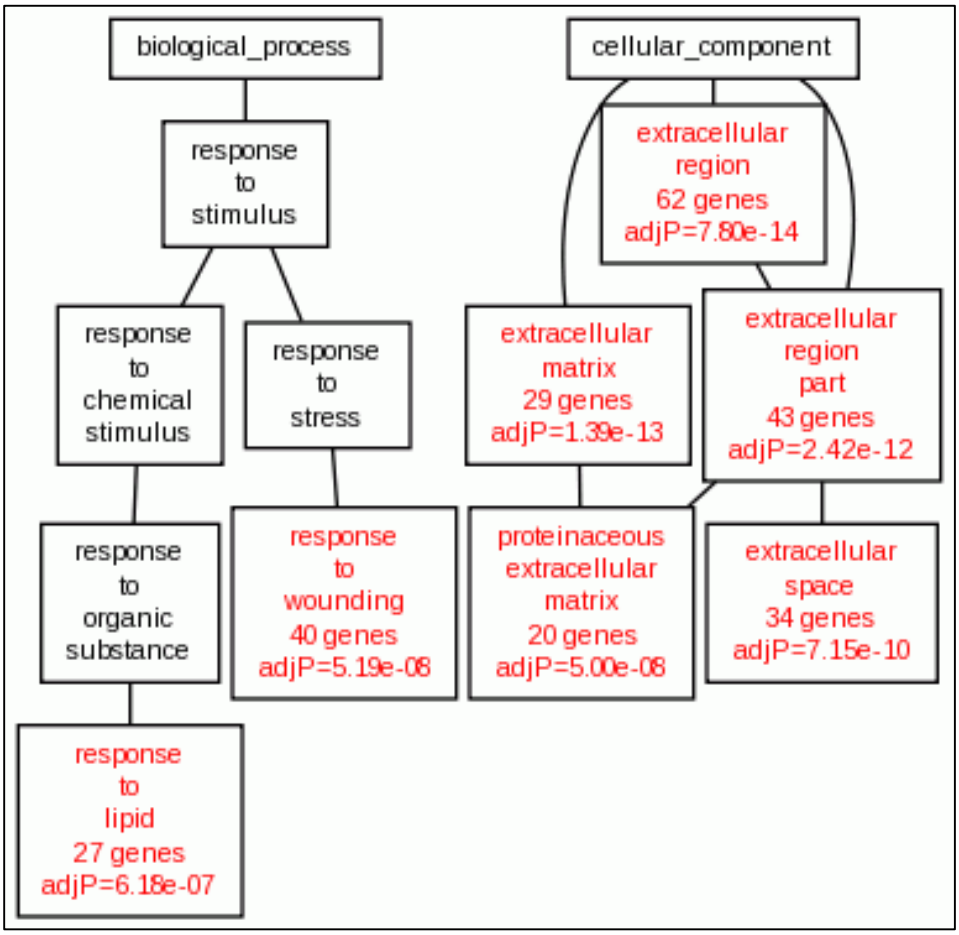


Figure 4.9. WebGestalt Directed Acyclic Graphs (DAGs) showing enriched GO categories under Biological Process, Molecular Function, and Cellular Component of genes with higher mRNA expression for MS compared to LW pigs by fold change. GO term enrichment related to lipids was detected.

4.3.1.4 Pathway analysis

A number of significant pathways were identified by Ingenuity® Pathway Analysis (IPA). However analyses were focused on gene expressions related to AT, lipid metabolism and energy production (Figure 4.10). Genes were prioritized for inclusion for the following reasons: (i) factors associated with food intake and energy expenditure which would influence adiposity, which in turn may be linked to performance in reproductive functions, such as *LEP*, (ii) nutritional compounds, such as vitamin A and D, calcium, with their receptors and binding proteins, that may affect deposition or release of lipid in AT, and (iii) factors involved in promoting growth in juvenile and growing animals. Icons in red and green are genes found within the DEG list, representing genes with higher or lower expression in LW compared to MS pigs respectively. Icons in white, orange and blue are genes not found in the DEG list, but are associated with functions related to pathways of interest. Genes or molecules coloured in orange are predicted to activate downstream targeted molecules, while the ones in blue are predicted to inhibit downstream genes. Solid lines connecting the genes and molecules indicate direct activation or inhibitory interactions, whereas broken lines indicate the presence of intermediate genes or molecules between the two, but not shown in the network diagram. Likewise, orange lines indicate an activation relationship, and lines in blue are inhibitory, while the ones in yellow indicate inconsistent relationship to the state of downstream gene or molecule with reference to Ingenuity® Knowledge Base. Shape of the end of lines also depict the predicted relationship between genes and molecules, arrow symbolise activation, whilst a short perpendicular line indicate inhibition.

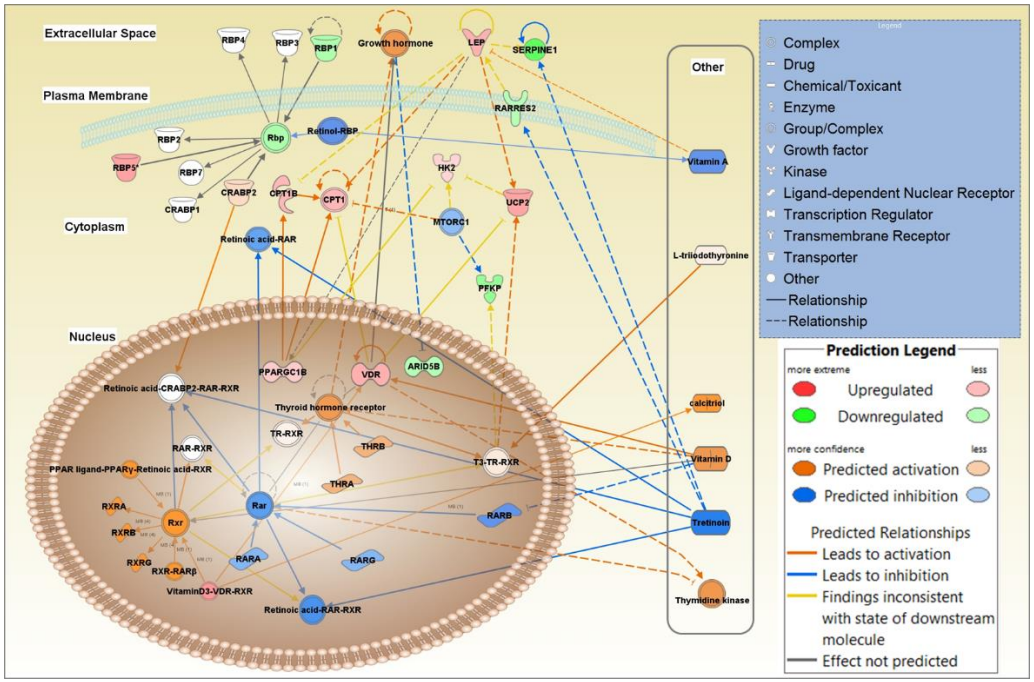


Figure 4.10. Ingenuity® Pathway Analysis highlighted interacting networks of genes of interest between MS and LW pigs. Diagram was overlaid with DEG list comparing MS and LW pigs, where gene expression referred to that of LW. Other genes of importance related to adipogenesis, lipogenesis, lipid metabolism, energy expenditure, and exogenous materials, such as nutritional compounds: vitamin A and D were added. The general function of genes and molecules are represented by differently shaped icons and the gradient of colours indicate the intensity of gene expression or the confidence of prediction. Icons in red and green represent genes with higher and lower mRNA expressions in the DEG list, while the ones in white, orange and blue were genes not found in the list, but are associated with functions related to pathways of interest. Solid lines connecting the genes and molecules indicate direct interactions, whereas broken lines indicate presence of intermediate genes or molecules between the two, but not shown in the pathway network. An arrow head at the end of a line depicts activation whilst a short perpendicular line indicates inhibition activity of the gene.

4.3.2 Validation by quantitative reverse transcription PCR

Several genes of interest were selected in accordance with their functions and activities in regulating reproductive activities, metabolism and energy expenditure, such as *LEP*, *UCP2*, *VDR*, and *CRABP2*. RNA-seq results indicated lower expression of *LEP,* uncoupling protein 2 (*UCP2*)*, VDR* and cellular retinoic acid binding protein 2 (*CRABP2*) in MS as compared to LW (Table 4.5). The primary reason these genes were chosen for further investigation is because modification of diet formulation in terms of energy level and nutritional content such as fat, protein, vitamins and minerals, is achievable and likely to be implementable in a shorter timeframe, compared to manipulation of gene expression and selective breeding. The output from this work will facilitate the process of knowledge transfer from research to application as improvement of fertility in production animal will have a positive impact on the livestock industry.

Comparison of qRT-PCR results between MS and LW revealed statistically significant differences for expression of *UCP2*, *VDR*, and *CRABP2* (FDR < 0.0001); (Figure 4.11), and validated the RNA-seq results. However, the expression of *LEP* did not differ significantly between MS and LW. This may be due to the level of expression differences between samples from these two breeds were not sufficiently high to be accurately quantified by qRT-PCR.

Table 4.5. Gene expression FC between MS and LW pigs through RNA-seq. *LEP, UCP2, VDR* and *CRABP2* were genes of interest selected for qRT-PCR validation due to their functions and activities in regulating reproductive activities, metabolism and energy expenditure. These genes had higher mRNA expressions in LW compared to MS pigs.

Genes	LW *vs* MS Log FC (FDR < 0.05)
Cellular retinoic acid binding protein 2 (*CRABP2*)	1.12
Leptin *(LEP*)	2.34
Uncoupling protein 2 (*UCP2*)	2.70
Vitamin D receptor (*VDR*)	2.41

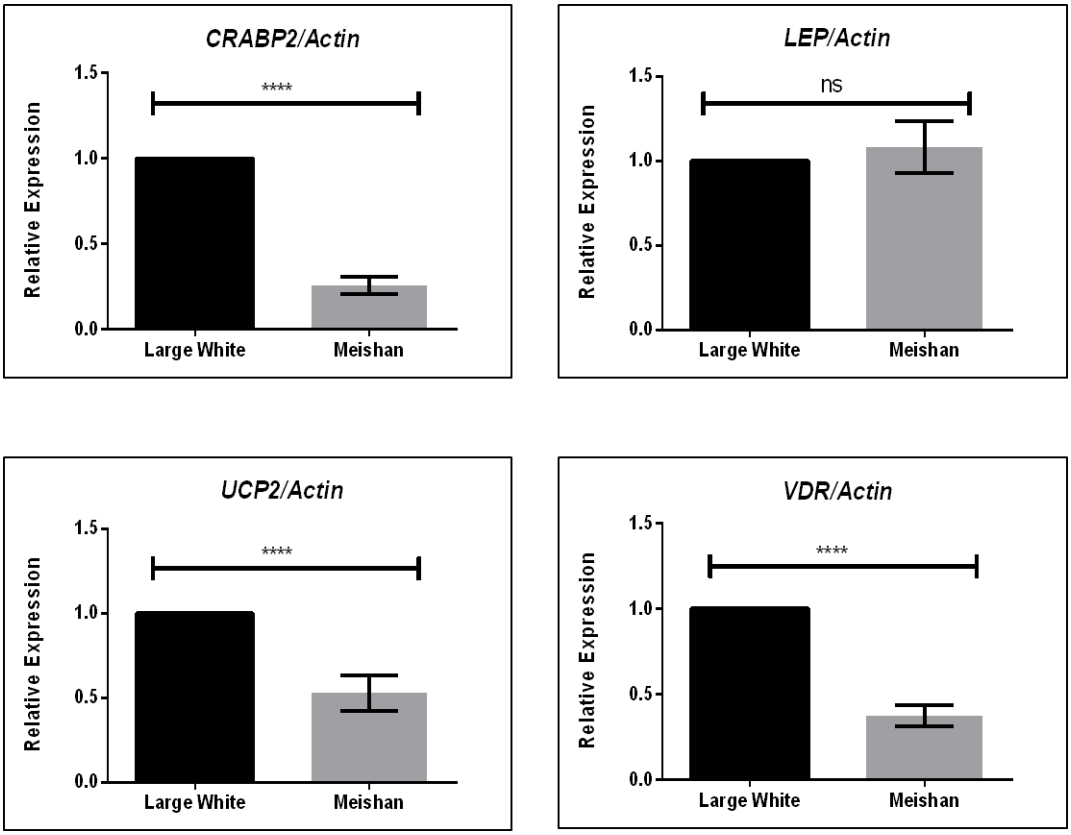


Figure 4.11. qRT-PCR validation of mRNA expression between MS and LW pigs. qRT-PCR results for *CRABP2, VDR,* and *UCP2* were consistent with RNA-seq expression. However, *LEP* expression did not differ significantly between the breeds through qRT-PCR. Mean values ± SEM. Level of significance: ns = non-significant, **** = $p < 0.001$. MS n = 4, LW n = 6.

4.4 Discussion

4.4.1 Identification of differentially expressed genes

RNA-seq was used to interrogate the transcriptome of AT in pigs, as described for the cattle in the previous chapter. The latest *Sus scrofa* genome assembly available, produced by Swine Genome Sequencing Consortium (SGSC); (Sscrofa10.2.71, August 2011) released in May 2012, and last updated in February 2014, was used for RNA-seq in this study (Ensembl, 2015c).

The lower raw read numbers of LW2 may be of concern as it was between 13 million to 30 million reads less compared to other samples (MS1, MS2, LW1), thus the number of mapped reads to reference genome was lower as well (Table 4.3). In general, it is advisable to take equal sequence depth, defined as the number of reads returned from a given sequencing experiment, between samples in order to support accurate statistical analysis of data generated (Tarazona et al., 2011). However, sequencing output and mapped reads of LW2, as well as other samples, have exceeded the minimum criteria stated in Standards, Guidelines and Best Practices for RNA-seq v1.0 released by the Encyclopedia of DNA Elements (ENCODE) Consortium (ENCODE, 2011), in which it calls for a minimum of 2 biological replicates, with 30 million paired-end reads of length over 30 nucleotides, of which 20-25 million reads are mappable to the genome or known transcriptome. Therefore, the sequence depth of replicates in the current study is considered to be moderate yet sufficient for detection of DEG between experimental subjects according to the ENCODE guideline (ENCODE, 2011).

From 21,630 coding genes defined in the Ensembl database, RNA-seq detected expression of 11,733 genes, and among these, 440 were found with significant DE between the breeds (FC $\geq$ 2, FDR < 0.05). The smear plot and volcano plot (Figures 4.4 & 4.5) shows a high concentration of detected genes within the region denoted as log FC between 1 to -1, corresponding to FC = 2 in expression. Thus, the proposed cut off for genes with FC $\geq$ 2 includes genes underpinning breed specific physiological differences in AT.

4.4.2 Gene ontology term enrichment annotation

The GO term enrichment annotation was used to gain a general overview of biological processes and molecular functions that were overrepresented in DEG (Rhee et al., 2008) between MS and LW. However, it should be noted that most GO analysis tools are based on knowledge bases populated by literature derived from direct or indirect experimental and computational evidence on human, and experimental subjects such as mice and rats. The WebGestalt mammalian database was limited to human, mouse, rat and dog, therefore pig Ensembl gene identifiers were translated into orthologous human Ensembl gene identifiers before analysis was performed. From 440 pig Ensembl gene identifiers, 329 were successfully translated to orthologous human identifiers with one to one conversion. Therefore, in both GO analyses, over 100 genes were not included. Thus one limitation to this analysis is the exclusion of approximately a quarter of DEG may lead to under-representation or even total omission of biological processes and functions that could be of significance, in addition to the relative scarcity of information on pig. It is

possible that variations may arise between orthologous genes after the point at which the species diverged. Although it is most likely that orthologous genes maintained transcriptional responses associated with biological processes that are evolutionarily conserved between species (Kristiansson et al., 2013).

Genes related to muscle development and function had higher mRNA expression in AT of 7-day old LW compared to MS pigs (Table 4.3). This raises the question whether AT possessed molecular properties similar to muscle. This is especially true for LW, as this breed has been reported to have a higher muscle growth potential than MS (White et al., 1995). AT is now regarded as an endocrine organ, releasing multiple adipokines that affects various physiological functions in the body (Trayhurn and Beattie, 2001). It has been proposed that AT and muscle may derive from a common mesenchymal precursor (Gesta et al., 2007, Sanchez-Gurmaches and Guertin, 2014). Moreover, MSCs or MSC-like cells isolated from various organs and tissues are able to differentiate into myocytes, osteoblasts, adipocytes, and chondrocytes *in vitro* (Sakurai et al., 2006, Phinney and Prockop, 2007, Billon et al., 2008). From these observations, one may speculate about the possibility that AT exerting transcriptional signals similar to muscle, and/or secretion of factors which promote muscle development. Insulin-like growth factor 2 (*IGF2*) and acid labile subunit (*ALS*) expression was higher in LW compared to MS. Paredes and colleagues (2013) found that IGF2-specific mRNA expression was elevated in high growing piglets, with a higher number of myofibre and a greater degree of myofibre hypertrophy. In humans and mice, ALS deficiency was reported to cause marked reduction of circulating insulin-like growth factor 1 (*IGF1*) and insulin-like growth factor binding protein 3 (*IGFBP3*) which lead

to mildly retarded growth (Domene et al., 2011). These observations appeared to agree with the enhanced growth rate of LW compared to MS, and it may be related to the heightened capacity for muscle accretion of LW pigs.

The GO term enrichment of genes with higher mRNA expression in MS pigs are related to wound healing and inflammatory reactions (Figure 4.9). This may be explained by the chronic low-grade inflammation known to occur in AT with increased adiposity, due to increased secretion of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), lipopolysaccharides, interleukin-6, and, interleukin-1β, together with recruitment and infiltration of macrophages, T cells, natural killer cells, and immature dendritic cells (Cildir et al., 2013). Furthermore, *RARRES2* mRNA expression was observed to be elevated in obese humans (Chakaroun et al., 2012), due to infiltration of pro-inflammatory cytokines into AT (Rourke et al., 2013). This suggests that AT of MS has a higher adiposity level compared to LW pigs.

4.4.3 Validation by quantitative reverse transcription PCR

The DE of *LEP* identified by RNA-seq between breeds was not validated by qRT-PCR (Figure 4.11). This may be explained by low mRNA expression level in the adipose samples, as piglets at 7-day of age may not have accumulated sufficient amount of fat to express *LEP*. Moreover, it has been reported that next generation sequencing (NGS) has similar or slightly better sensitivity, and more accurate quantitative and qualitative evaluation of mRNA content within a cell or tissue compared to standardized qRT-PCR, even although the latter was considered the “gold standard” to quantify gene expression (Brooks et al.,

2011). This can be attributed partly to the higher number of cells analysed by NGS achieved by higher input cell amounts (Brüggemann et al., 2013), and the normalisation of gene expression across the transcriptome as opposed to one or two reference genes (Kozera and Rapacz, 2013). However, given the significant cost associated with NGS and the comparative ease of qRT-PCR, qRT-PCR will remain an important technique in the years to come (Kozera and Rapacz, 2013, Liu et al., 2012a).

4.4.4 Genetic interaction and function of selected genes

As described in previous sections, MS sows have been reported to outperform the LW reproductively, with sexual maturity at an earlier age, lower embryonic mortality, higher ovulation rate, a higher numbers of live births, and neonatal survival in MS compared to LW pigs (Bolet et al., 1986, Bazer et al., 1988b, Bidanel et al., 1990, Hunter et al., 1993, White et al., 1993b, Haley et al., 1995, Lee et al., 1995, White et al., 1995). The higher percentage of body fat in the MS compared to that of the LW pigs may be an important factor, as AT has been reported to be pertinent in regulation of the HPG axis, although the mechanism(s) underpinning this phenomenon is yet to be fully established. In part, the goal of the current study is to determine molecular signatures of AT that may influence reproductive efficiency in animals.

One of the main genes of interest in this study was *LEP*. *LEP* is mainly secreted by white adipocytes, and it has a role in the regulation of body weight by inhibiting food intake and increasing energy expenditure to maintain constancy of the adipose mass (Friedman and Halaas, 1998). It has also been

proposed to be involved in signalling the HPG axis to initiate the cascade of reproductive hormone production (Chan and Mantzoros, 2001). Consistent with this, *LEP* levels increased with age in pigs, most likely connected with elevated levels of adiposity with age (Barb et al., 2001, Mitchell et al., 2001). In this study, *LEP* expression was higher in LW compared to MS in RNA-seq. *LEP* expression in white adipose tissue (WAT) and brown adipose tissue (BAT) was found to be inhibited by all-*trans* retinoic acid (ATRA) treatment in mice, resulting in marked reduction of AT adiposity due to increased energy expenditure that may involve the uncoupling proteins, as well as a lower appetite (Kumar and Scarpace, 1998, Bonet et al., 2000). Therefore, the level of *LEP* mRNA expression in LW could be lower than MS due to the inhibitory action of ATRA induced by elevated activation of *CRABP2*. However in this case, *LEP* level in MS were lower than LW, this may suggest that the MS pigs have not reached the level of adiposity necessary to initiate an elevated release of LEP due to the slower growth rate in MS compared to LW, thus promoting further adipose deposition in this breed.

The AT development in LW pigs compared to that of MS, may also be suppressed by higher *CRABP2* expression in LW (Figure 4.10), as this gene has been reported to inhibit preadipocyte differentiation by enhancing the adipogenic inhibitory ability of ATRA (Berry et al., 2010). ATRA has been reported to enhance fat catabolism and energy expenditure, leading to a reduction in adipocyte differentiation and adiposity by enhancing lipolysis, as well as decreasing lipogenesis in WAT (Mercader et al., 2006, Berry and Noy, 2009). An important function of CRABP2 is to facilitate ATRA binding to its receptor complex, increase stability of the ATRA nuclear complex receptors to

the promoters of their target gene (Delva et al., 1999, Vreeland et al., 2014, Trasino et al., 2015). CRABP2 binds to ATRA in the cytosol, then binds with RAR in the nucleus, forming a complex through which ATRA move from CRABP2 to RAR (Takase et al., 1986, Gaub et al., 1998, Dong et al., 1999, Trasino et al., 2015). Increased expression of *CRABP2* stimulates the transcriptional activity of *RAR* by directly interacting with the receptor, hence facilitating the formation of the active RAR-ATRA complex which in turn elevated intracellular availability of intracellular ATRA (Dong et al., 1999, Budhu and Noy, 2002, Manolescu et al., 2010, Vreeland et al., 2014).

Furthermore, vitamin A-deficient diet feeding has been demonstrated to lead to a marked increase in adiposity and to a small increase of body weight (Ribot et al., 2001). Treatment with ATRA reversed the increase of adiposity and body weight, as this was correlated with downregulation of *PPARγ* expression in AT (Ribot et al., 2001), a master regulator driving adipocyte differentiation in adipogenesis (Rosen et al., 2002), also critical for maintenance of differentiated murine adipocytes *in vivo* (Imai et al., 2004, Schupp et al., 2009). Reports indicated that *CRABP2*, the coactivator for the RAR and RXR heterodimer, regulates ATRA transcription through RAR, inhibits adipocyte differentiation, and thus reduces adipogenesis (Berry et al., 2010).

Retinol binding protein 5 (*RBP5*) mRNA expression was also elevated in LW compared to MS pigs (Figure 4.10). RBP5 or cellular retinol-binding protein type III (*CRBP3*) is a member of intracellular lipid-binding proteins. In the cytosol, RBP5 binds retinol, the precursor of retinyl ester and the active metabolite ATRA (Zizola et al., 2008). RBP5 mediates retinol uptake from

circulating retinol-RBP and helps in facilitating retinol oxidation to ATRA (Vogel et al., 2001), thus logically this will increase the availability of ATRA intracellularly and impede adipogenesis. However, studies by Zizola and colleagues (2008) showed that RBP5-KO mice exhibit decreased appetite, fat mass, adipocyte lipid content and energy expenditure compared to wild type mice. They also found that levels of retinol and retinyl ester in AT from RBP5-KO and wild-type mice are similar, this suggests that actions of RBP5 are most probably independent of its binding to retinol, as well as independent of retinoid metabolism. *RBP5* is a *PPARγ* target gene, and *RBP5* expression is regulated by *PPARγ in vitro* and *in vivo* and was implicated to be involved in lipid metabolism (Zizola et al., 2008). However, the increased of *RBP5* expression in LW did not correspond with elevated *PPARγ* expression. Thus, expression of *RBP5* may be induced by other factors in the current study.

One of the mechanisms by which ATRA affects AT, which was described in mice, is the remodeling of low oxidative WAT so that it resemble BAT, with high oxidative capacity, increased *UCP1* expression, reduction in size of adipocytes, and multilocular lipid vacuoles, instead of the typical large unilocular lipid droplet of WAT (Mercader et al., 2006, Berry and Noy, 2009). It is now established that this is due to presence of beige adipocytes in the WAT, together with white adipocytes. Beige adipocytes are a distinctive cell type from white and brown adipocytes. They resemble white fat cells with extremely low basal expression of *UCP1*, but when stimulated, they respond with high *UCP1* expression and enhanced respiration rates similar to the brown adipocytes (Wu et al., 2012). BAT was previously reported to be absent in pigs (Trayhurn et al., 1989), thus *UCP1* which is exclusively expressed in

BAT was also believed to be absent in this species (Berg et al., 2006). However in more recent studies, presence of BAT was demonstrated in histological sections of AT from pigs (Attig et al., 2008), and expression of *UCP1* was later confirmed through qRT-PCR and immunohistochemistry (Mostyn et al., 2014). However, results from the latter study suggests that the AT in the piglet is beige, rather than the classical brown depot (Mostyn et al., 2014), as it expresses genes that are markers of both BAT and WAT (Wu et al., 2012, Pope et al., 2014). *UCP1* expression was not detected in RNA-seq dataset in this study as the reference genome has yet to include *UCP1*.

UCP2 is a mitochondrial protein, and was reported to have the highest level of expression in WAT compared to other tissues in adult human (Fleury et al., 1997). Research suggests an important role for *UCP2* in thermogenesis, energy metabolism, and obesity (Fleury et al., 1997, Gimeno et al., 1997). The higher expression of *UCP2* observed in LW correlated the results from previous studies and suggests a possible cause of lower adipose deposition in LW. An increase in adiposity promotes expression of *UCP2* (Kozak and Harper, 2000), thus inducing fat oxidation and inhibition of lipogenesis (Andrews et al., 2010).

Furthermore, *UCP2* expression was shown to be downregulated by treatment of human adipocytes *in vivo* with calcitriol, the biologically active form of vitamin D. Calcitriol acts via *VDR*, causing elevation of the intracellular calcium level in human adipocyte culture and leading to suppression of *UCP2* (Shi et al., 2002). Expression of uncoupling proteins (*UCP1* and *UCP2*) was elevated in the WAT of vitamin D deficient rats, leading to higher energy expenditure,

and thereby a lean phenotype (Bhat et al., 2014). Also, it was noted that calcium supplementation could reverse the depletion of AT caused by vitamin D deficiency in rats (Bhat et al., 2014). On the other hand, vitamin D, acting via the VDR-retinoid-X-receptor complex, was observed to stimulate *LEP* mRNA expression & secretion in wild type AT and cultures (Kong et al., 2013). In the present study, VDR expression was higher in LW, yet *UCP2* expression was also elevated. Thus, it seems that increased *UCP2* and *VDR* expressions in AT of LW suggests that AT expansion may be suppressed in LW compared to MS, and energy might be channelled towards muscle development.

Peroxisome proliferator activated receptor γ coactivator 1β (*PPARGC1β*) expression was higher in LW compared to MS pigs (Figure 4.10). Human patients with pheochromocytoma, a tumour associated with expansion of BAT and induction of *UCP1* gene expression in peri-renal fat, are reported to have elevated *PPARGC1β* mRNA expression in peri-renal AT (Esterbauer et al., 1999). Again, this is consistent with lower adiposity potential of LW pigs. It could also imply that AT in LW was less mature than that of MS pigs, as *PPARGC1β* gene expression increased in parallel with pro-adipogenic genes such as adiponectin (*ADIPOQ*) and *FASN* in human preadipocyte cultures during adipocyte differentiation (Moreno-Navarrete et al., 2014).

Higher mRNA expression of *PPARGC1*, and carnitine palmitoyltransferase 1B (CPT1B) was observed in LW compared to MS pigs (Figure 4.10). Mouse *Ppargc1* has been reported to enhance expression of *UCP1* through stimulation of mitochondrial oxidative metabolism (Puigserver and Spiegelman, 2003). Heterogynous knockout of *CPT1B* in mice resulted in

exacerbated mitochondrial abnormalities and myocardial lipid accumulation with elevated triglycerides and ceramide content (He et al., 2012). Therefore, higher mRNA expression of *PPARGC1β*, and *CPT1B* in LW pigs may induced increased expression of carnitine palmitoyltransferase I (*CPT1*) complex (Figure 4.10), which could boost mitochondrial activities and lipolytic metabolism of AT in LW pigs.

Lower mRNA expression of AT rich interactive domain 5B (*ARID5B*), also known as modulator recognition factor 1-like (*MRF1*-like) and modulator recognition factor 2 (*MRF2*), was detected in AT of LW compared to MS pigs (Figure 4.10). Murine 3T3-L1 preadipocytes with knockdown of *Arid5b*, was reported to exhibit decreased expression of CCAAT/enhancer binding protein α (*C/EBPα*) and *PPARγ*, thus adipogenesis was inhibited. Furthermore, mature 3T3-L1-derived adipocytes with knockdown of *Arid5b* have enhanced lipolysis and triglyceride synthesis, which caused a significant increase in the ratio of glycerol release to free fatty acids (FFAs); (Yamakawa et al., 2008, Yamakawa et al., 2010). This may explain the severe reduction of BAT and WAT lipid accumulation of neonate and adult mice with *Arid5b* homogenous knockout (Whitson et al., 2003). Thus, lower mRNA expression of *ARID5B* in RNA-seq from the current study is consistent with the lower adiposity seen in LW compared to MS pigs.

Retinoic acid receptor responder (tazarotene induced) 2 (*RARRES2*) mRNA expression was lower in LW compared to MS pigs (Figure 4.10). *RARRES2*, also known as chemerin, is an adipokine that appears to be primarily produced by WAT (Goralski et al., 2007, Fatima et al., 2014). Increased adiposity was

shown to elevate *RARRES2* mRNA expression in AT and serum concentration in human (Sell et al., 2009, Chakaroun et al., 2012) and mice (Goralski et al., 2007). *RARRES2* was reported to promote adipocyte differentiation and metabolism by binding to and activating the G protein-coupled receptor, chemokine like receptor-1 (*CMKLR1*); (Muruganandan et al., 2011, Ernst et al., 2012). *RARRES2* was reported to significantly mediate angiogensis to a similar extent as vascular endothelial growth factor (*VEGF*); (Bozaoglu et al., 2010) in order to increase the required blood supply for hyperplasia and hypertrophy of AT mass (Rourke et al., 2013). Knockdown of chemerin or *CMKLR1* expression or antibody neutralization of secreted chemerin protein severely reduce differentiation of murine MSCs into mature adipocytes (Muruganandan et al., 2011), and *CMKLR1* knockout mice have reduced body mass and adiposity, and are resistant against diet-induced obesity (Ernst et al., 2012). Thus, lower *RARRES2* expression in the LW indicates a lesser degree of AT mass growth in LW compared to MS pigs.

RNA-seq results suggest there was a higher adipogenic and lipogenic capacity in MS pigs compared to LW attributable to the lower expression of *CRABP2*, *LEP, UCP2*, and *VDR*. Lower expression of *LEP* and *VDR* in MS pigs suggests enhanced adipocyte energy uptake and lipid deposition in this breed compared to LW, whereas lower *CRABP2* and *UCP2* expression may lead to reduced FA oxidation, lipolytic activities, and energy expenditure. Collectively these transcriptional networks may cooperate to promote adiposity in MS pigs. This is further supported by the higher mRNA expression of *RARRES2*, and iroquois homeobox 3 (*IRX3*) in MS compared to LW pigs (log FC = 1.40, and 1.59 respectively, FDR < 0.05). Both of these genes were previously reported

to be highly expressed in adipocytes and have also been implicated in enhancing glucose uptake and reducing basal metabolic rate (Goralski and Sinal, 2009, Rourke et al., 2013, Gorkin and Ren, 2014, Smemo et al., 2014).

4.5 Conclusion

From these results, it is likely that MS have a lower energy expenditure than LW pigs, which could promote adipogenesis and lipogenesis over time and thereby encourage adiposity. In contrast LW pigs tend to perform better in terms of muscle growth and this is reflected in the increased expression of muscle-associated genes. This indicates that transcriptional signals from AT of these two breeds are significantly different underpinning the physiological distinction between the two. Results imply that nutritional intervention could be a practical and feasible way to affect adiposity in livestock through feed formulation, and impact on reproductive performance may be more readily achieved in shorter time frame than would be accomplished by a selective breeding programme. Although RNA-seq results did not exhibit mRNA expression directly related to reproduction, this was understandable as samples were obtained from piglets that have yet to reach sexual maturity. Further studies are therefore required to analyse mRNA expression of AT from sexually matured animals. In addition, this study was limited to examination of transcriptomic expression. Experiments to investigate protein levels induced by mRNA expression through immunohistochemistry and Western blotting will help to further confirm results obtained thus far.

Chapter 5 Molecular Characterisation of Adipose Tissue of African Elephants

5.1 Introduction

5.1.1 Classification, geographical range and threats faced by African elephants

A total of 182 species and subspecies and 44 genera have been identified under the Proboscidae order (Sanders et al., 2004, Shoshani, 2006). However today there are only 2 surviving genera: *Loxodonta* and *Elephas* (Sanders et al., 2004, Shoshani, 2006). African elephants are the only living member of animals in the order Proboscidae under the family Elephantidae found on the African continent. Two species have been identified through morphological comparison, habitat distinction, and phylogenetic analysis: the African savannah elephant (*Loxodonta africana*), and the African forest elephant (*Loxodonta cyclotis*); (Roca et al., 2001). However a later study utilising mitochondrial DNA sequences and nuclear microsatellite markers proposed that there may be a third species distributed throughout western Africa. Results suggested that the West African Elephants are genetically distinctive from both the forest elephants at central Africa, and savannah elephants found in central, eastern and southern Africa, with a divergent in evolutionary trajectory occurring more than 2 million years ago (Eggert et al., 2002). Currently, African elephants are listed as vulnerable by the IUCN (Blanc, 2008), which means they are currently not critically endangered or endangered, but are facing a high risk of extinction in the wild in the medium term future if the population cannot be sustained (IUCN, 1994).

The African elephant is the largest terrestrial mammal, with males recorded at a height of 3.3 metres at the shoulder, and 6 tonnes in weight (WWF, 2015), while the females are considerably smaller with an average weight of 2,000 to 4,000kg, and average height of 2.3 – 2.7m at the shoulder (Schmidt, 1986). The current wild African elephant population is estimated to be 633,005 according to surveys conducted in December 2013, a decline of 8.5% from the previous census record of 691,960 in 2006. However this number may be as low as 419,000, since not all populations across the continent could be accounted for, and figures were extrapolated from previous data (AfESG, 2013). Poaching is an immediate and direct threat to the African elephant, and incidence has increased across all regions since 2007, after a decline in latter part of the 1990s from the high incidence of poaching that occurred between 1970 and 1990. The highest level of poaching was recorded in 2011, when it was estimated that 7.4% of the total elephant population was illegally killed, and this translated to approximately 34,000 animals. This trend is expected to continue (UNEP et al., 2013, Wittemyer et al., 2014). Although ivory is the main reason for poaching, elephant meat and skin trade is also increasing (Randolph and Stiles, 2011).

As with many wildlife species, the African elephant also faces the threat of home range and habitat loss due to human population expansion and conversion of forest and grassland for agricultural use. These are believed to be the most significant long term threat to the survival of the species (WWF, 2013, WWF, 2015). The total range of the African elephant was estimated at 26% of the continent's total land area in 1995 (Said et al., 1995), and this has dropped to only 11% by 2007 (Blanc et al., 2007). This inevitably led to

fragmentation of the elephants' habitat and may impede their access to natural resources. With substantial habitat loss coupled with a marked escalation of poaching for ivory, the wild population will not be able to recover sufficiently to sustain the current ivory consumption, and it is believed that the African elephant is at risk of extinction in the wild within the next few decades (Wittemyer et al., 2014). It is hoped this will be countered by the existence of protected areas in Eastern and Southern Africa, although evidence indicates that some illegal harvest of ivory has occurred even in these protected areas (UNEP et al., 2013).

5.1.2 Captive African elephants

5.1.2.1 Utilisation of captive population

Captive populations of elephants have been recorded since the beginning of early civilisations. These animals served as prestige symbols of royalty and imperial power, they were used for warfare, as well as for hunting and ceremonial rituals. In modern times, elephants have been used as show animals in circuses, major attraction in zoos and safaris, and provide draught power in some countries, especially in the logging industry (Wylie, 2008). However, African elephants were not captured and utilised as much as the Asian elephants. This may be due to the lack of demand, but also these animals have been reported to be more difficult to train (Clubb and Mason, 2002). Historical records indicate that African elephants were mainly used for military purposes. Of these, the most famous example was that of Hannibal, a Punic Carthaginian military commander, who incorporated African elephants

in his army and triumphed against the Roman legion circa 219 BC (Iversen, 1996, Wylie, 2008).

In more recent years, African elephants have been trained to carry tourists in national parks rather than using motorised vehicles. This has provided a unique experience for visitors, increased park revenue and local employment, and reduced vehicle-related erosion and pollution in the parks (Iversen, 1996). Elephants in zoos are no longer just a point of attraction, but are believed to play an important role in public education, scientific research, development of technologies (e.g. steroid hormone detection from faecal samples (Wasser et al., 1996), artificial insemination (Hildebrandt et al., 1999, Thongtip et al., 2009), professional training, and raising conservation funds (Smith and Hutchins, 2000). It is suggested that the presence of live animals in educational programmes increases engagement of the public's interest, and therefore there is a greater opportunity to increase awareness of the threats faced by the wild population (Saunders and Young, 1985). Zoos have also been able to raise funds in support of field conservation and habitat protection (Smith and Hutchins, 2000). Scientific research conducted *ex-situ* can supplement field studies and technologies can be developed in a more controlled environment before trials are run *in-situ* (Hutchins and Conway, 1995). Also, results from scientific research and management methods in captive population can be shared with personnel involved in field conservation (Smith and Hutchins, 2000).

Nevertheless, upkeep of elephants in captivity has generated welfare concerns in the recent years with regards to capacity and enclosure size of the

captive facilities, availability of family herd to maintain social systems, restriction of movements due to chaining, risks of disease, environmental enrichment, diet, etc. (Clubb and Mason, 2002, Evans and Harris, 2008). Comparative studies of *in situ* conservation of habitat and wild population and *ex situ* breeding programmes revealed that per capita costs for effective *in situ* conservation of large animal such as the African elephant, are consistently lower than those maintained in captivity (Leader-Williams and Albon, 1988, Balmford et al., 1995). Thus, it has been suggested that investment in well-managed field-based initiatives could be more effective to conserve large wildlife species and their natural habitat, as well as benefitting the local communities than establishing *ex situ* breeding programmes (Balmford et al., 1995).

5.1.2.2 Reproductive challenges of captive African elephants

Captive breeding was not a major concern until the late 20th century as replacement animals were mostly acquired through importation from range countries, which often involved extrication of animals from the wild (Wiese and Willis, 2006). Because of the financial, logistical, and ethical concerns of capturing and importing wild elephants, as well as the restrictions on exportation of these animals from their countries of origin due to the threats and pressures on the local populations faced from international trade (CITES, 2013), it is believed to be imperative that the existing captive population is self-sustainable. Furthermore, as the wild population faces increasing pressure from poaching and habitat loss, captive populations may form a safeguard

against extinction of the species or subspecies (Wiese and Willis, 2006). Unfortunately, current reproductive output is unable to sustain the captive population, and needs to be more productive and effective (Olson and Wiese, 2000, Brown, 2014). However, *ex-situ* conservation efforts cannot replace the biodiversity which exists in nature, thus preservation of natural habitats is still essential (Rahbek, 1993, Balmford et al., 1995). The focus of the current study was on African savannah elephants (*Loxodonta africana*), thus the following discussion will be centred on this species.

There are about 1,000 captive African elephants globally, mostly in zoos. Most of these captive elephant populations are not self-sustaining due to high mortality and low birth rates (Brown, 2014). It was postulated that at the current birth rate in U.S. zoos, African elephants would cease to exist in zoos within the next 50 years, as the current population would have long since passed prime reproductive age (Wiese and Willis, 2006). The causes of low fecundity in African elephants are multifactorial, including low number of breeding bulls, fertile females and males are not housed together, the stress of translocation to bring breeding animals together (Brown, 2000), behavioural issues such as mate incompatibility, suppression of oestrous cycles due to the hierarchy within the group (Freeman et al., 2009, Brown, 2014), and physiological factors such as ovarian cycle abnormalities, reproductive tract pathologies, gestational difficulties and bull subfertility (Brown et al., 1999, Brown et al., 2004, Proctor et al., 2010, Dow et al., 2011, Brown, 2014).

One of the physiological causes proposed for poor reproductive success in captive elephants is their excessive body fat (Clubb and Mason, 2002, Morfeld

and Brown, 2014). Captive female African elephants are reported to be heavier than their free-ranging counterparts (Ange et al., 2001). While there appear to be nutrient deficiencies in most zoo diets, either in the quantity of crude protein, vitamins or minerals, zoo diets are generally too high in energy content, thus animals are prone to obesity (Ange et al., 2001, Hatt and Clauss, 2006). This may be exacerbated by confinement in indoor enclosures over extended periods of time, which limits the amount of exercise accessible for zoo elephants (Clubb and Mason, 2002).

Nearly half of African female elephants in zoos exhibit abnormal ovarian cycles, either irregular cyclicity or acyclicity, and these conditions are strongly correlated with a high body mass index (UAB, 2014). It has been estimated that obesity affects about 40% of African elephants in captivity, and this may be major factor of infertility in these animals (Brown et al., 1999, Brown et al., 2004, Proctor et al., 2010, Dow et al., 2011, Brown, 2014). Furthermore, it was estimated that at least 70% of the non-cycling captive female elephants exhibit some form of ovarian or uterine pathology that potentially impedes successful breeding of these animals (Brown et al., 2004), although acyclicity issues in captive elephants do not appear to be entirely due to reproductive tract abnormalities (Proctor et al., 2010, Dow et al., 2011).

Indeed the high levels of obesity in zoo elephants may have a substantial impact on reproductive success in the population. Reports on Asian elephants indicated that overweight zoo dams are more likely to have a prolonged gestation period, and have higher frequencies of stillbirths, and these stillborn calves weighed more than live born ones, suggesting a further link with over-

nutrition of the mothers (Kurt and Mar, 1996, Mar et al., 2012). Live calves from overweight mothers are also born heavier and have a higher risk of calf mortality than calves from normal weight dams (Kurt and Mar, 1996).

Given the clear links between obesity and reproductive performance in elephants, it would be interesting to explore the transcriptional signals from adipose tissue (AT) in relation to reproductive functions in elephants. Research in human and domestic animals has indicated the importance of AT in reproductive performance and fertility (Frisch and McArthur, 1974, Armstrong and Britt, 1987, Frisch, 1987, Tatman et al., 1990, Wade and Schneider, 1992), and results presented in previous chapters on cattle and pigs, demonstrated differentially expressed genes (DEG) in animals with different adiposity level may influence reproductive performance of these species. Results from this study may provide baseline data for comparative studies between free range African elephants in the wild and their counterparts in captivity in terms of adipogenesis, lipogenesis, lipid metabolism, energy storage and expenditure, which in hope will help to improve management of African elephant in captivity and aid in enhancing fertility of these animals.

5.2 Materials and methods

5.2.1 Animals and samples

Elephant samples were obtained from management-organized culling operations in Save Valley Conservancy (SVC) in Zimbabwe between the years 2009 and 2011. Permits were granted from the Zimbabwe Parks and Wildlife

Management Authority (PWMA) to SVC to cull the animals and SVC gave the permission to use the samples for research. Sample collections were opportunistic, animals were not killed specifically for this study, and all permission was obtained from the relevant authorities. The project and acquisition of samples also received approval from the Research Directorate and the Ethics Committee at the School of Veterinary Medicine and Science, University of Nottingham.

5.2.2 RNA-sequencing and differential expression analysis

A total of 2 female and 1 male adult peri-renal adipose samples were sent for next generation sequencing (NGS); (Table 5.2). Work flow and protocols of total RNA extraction, RNA sequencing (RNA-seq), differential transcriptomic analysis, and validation of RNA-seq through quantitative reverse transcription PCR (qRT-PCR) were as described in Chapter 2. Trimmed sequences for the elephant adipose samples were aligned to the *Loxodonta africana* genome assembly released in March 2010 and updated in December 2011 (Loxafr3.0, INSDC Assembly, July 2009) by the Broad Institute, Massachusetts, USA. The genome assembly encoded 20,033 coding genes, 2,644 non-coding gene, 2,391 small non-coding genes, 253 miscellaneous non-coding genes, 568 pseudogenes, and 28,847 gene transcripts (Ensembl, 2015b).

5.2.3 Hierarchical clustering analysis

Hierarchical clustering analysis was performed using Cluster 3.0 as described in Chapter 2. To further determine the strength of support from the database on clusters formed, Pvclust (a R package programme) was used for clustering analysis, and p-values were generated for each node of the dendrogram (Shimodaira, 2004, Suzuki and Shimodaira, 2006). Pvclust calculates 2 types of p-values: AU (Approximately Unbiased) p-value and BP (Bootstrap Probability) value. The BP value was computed by normal bootstrap resampling, while AU p-value was computed by multiscale bootstrap resampling where probabilities were calculated for sets of bootstrap replicates with several sample sizes which may differ from that of the observed data. Therefore AU is believed to be a better approximation of unbiased p-values than BP (Shimodaira, 2002, Shimodaira, 2004). The p-values were then converted to percentages, and these numbers will represent the probability of the node formation on the dendrogram. Therefore, the greater the percentage value of the node, the higher the confidence that the clusters formed were accurate (Shimodaira, 2002).

5.2.4 Elephant primer design

Nucleotide sequence in FASTA format for mRNA of the gene was obtained through the National Center for Biotechnology Information (NCBI) gene reference sequence database (http://www.ncbi.nlm.nih.gov/gene). Human gene sequences were used as reference, as the human genome is the best studied and well annotated compared to other mammalian species (Table 5.1).

The FASTA file was submitted to UC Santa Cruz (UCSC) Genome Bioinformatics Site's BLAT search tool (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start) to identify comparable regions between human and elephant genomes. The nucleic acid sequences of putative elephant genes was derived, and intron-exon boundaries were identified. Matched bases in cDNA and genomic sequences were coloured blue and capitalised, light blue bases marked the boundaries of gaps in either sequence, while black and lower cased bases were mismatches. Primers were designed within the matched regions, although it was possible to have mismatched nucleotides in the middle of the sequence if unavoidable. The forward and reverse primers were designed to preferably contain 24 bases, with 50% of the nucleotides either guanine (G) or cytosine (C). Primers were designed to start and end on G or C, but not both, in order to prevent the primer ends from annealing to each other. Also, forward and reverse primers were approximately 150 to 200 bases apart. These criteria ensured optimised annealing of cDNA strands to the targeted region, and primers would be likely to span over two exons, thus precluding amplification of genomic DNA.

Primers sequence were checked using the UCSC *In-Silico* PCR tool (http://genome.ucsc.edu/cgi-bin/hgPcr). An output file in FASTA format containing nucleotide sequences that lay between the primer pair was returned. The FASTA header depicted the chromosome in which the primers were located, and also showed the number of bases between primers. If the number of bases between primers from the output file was larger than that established during the initial design process described above, this confirmed that the primers spanned across the exon-intron junction. The FASTA data

was capitalised in areas where the primer sequence matched the genomic sequence and was in lower-case elsewhere.

Table 5.1. List of designed primers and GenBank accession number for genes of interest in African elephants.

Gene name	Official symbol	Primer sequence	GenBank accession number
Actin, β	*ACTB*	F: 5-CCTGAGCGCAAGTACTCAGTGTGG-3 R: 5-ttttgtcaagaaaaggtgtaacgc-3	NM_001101.3
ELOVL fatty acid elongase 5	*ELOVL5*	F: 5-CAACCACCAGATCACGGTCCTGCA-3 R: 5-ccctgagtgatgtacttcttccac-3	NM_001242828.1
Fatty acid synthase	*FASN*	F: 5-GGGCTACAGCATGGTGGGCTGCCAG-3 R: 5-cttgcaggtgccctcggggctgagc-3	NM_004104.4
Leptin	*LEP*	F: 5-CAAGGTCTCCAGGCCACTGGCC-3 R: 5-TCACCAGGATCAGTGACATTTCACAC-3	NM_000599.3
Nuclear receptor subfamily 2, group F member 2	*NR2F2*	F: 5-AGTACAGCTGCCTCAAGGCCATAG-3 R: 5-gaaaaacaattgctctatgactga-3	NM_001145155.1
Peroxisome proliferator-activated receptor, γ	*PPARG*	F: 5-GGAGCCCAAGTTTGAGTTTGCTGT-3 R: 5-aaggcttgcagcaagttttcctgg-3	NM_005037.5
Retinoid X receptor α	*RXRA*	F: 5-CGCCCACCCCTCAGGAAACATGGC-3 R: 5-agtccttgttgtcgcggcaggtgt-3	NM_001291920.1
Stearoyl-CoA deaturase (δ-9-desaturase)	*SCD*	F: 5-CTTCTCTCACGTGGGTTGGCTGCT-3 R: 5-cactgttcaccagccaggtggcat-3	NM_005063.4

5.2.5 Determination of the sex of the elephant samples

Because the elephant samples were collected under difficult field conditions, it was important to confirm the sex of the elephant from which each sample was taken. In order to do this, each sample was tested for the presence of the sex determining region Y (*SRY*) gene, found on chromosome Y, through PCR. The SRY protein acts as a transcription factor that initiates male sexual development during the fetal stage. As SRY expression is not detectable in adults, DNA extracts of AT were used instead of RNA.

5.2.5.1 DNA extraction

DNA was extracted from AT of both sexes using DNeasy® Blood & Tissue Kit (Qiagen, Crawley West Sussex, UK). A total of 50mg AT was finely diced, and placed in a 1.5mL microcentrifuge tube. Lysis of AT was achieved by overnight incubation with 180µL Buffer ATL and 20µL proteinase K at 56°C in a water bath (SUB Aqua Pro, Grant Instruments Ltd., Cambridge, UK). Samples were removed from the water bath and vortexed (Evolution ZX FB15012 TopMix, Fisher Scientific, Loughborough, UK) for 15 seconds before proceeding with the following steps at room temperature. A volume of 200µL Buffer AL was added to the samples, followed by 200µL ethanol (96–100%). Samples were vortexed immediately after addition of each reagents to ensure thorough mixing, and a homogenous solution was obtained.

The mixture was then pipetted into a DNeasy Mini spin column placed in a 2mL collection tube, and centrifuged at 6,164g for 1 minute using a Heraeus PICO17 centrifuge (Thermo Scientific, Portsmouth, USA). DNA was expected to selectively bind to the DNeasy membrane as contaminants passed through, thus both the flow-through and collection tube were discarded, and the spin column was placed in a new 2mL collection tube. Two wash steps followed to further remove contaminants. First, 500µL Buffer AW1 was added, and the spin column was centrifuged at 6,164g for 1 minute. Then, 500µL Buffer AW2 was added, and the spin column was centrifuged for 3 minutes at 18,879g. After each wash step, the flow-through and collection tube were discarded. The spin column was placed in a new 2mL collection tube after the first wash, and transferred to a new 1.5mL microcentrifuge tube after the second wash.

DNA was eluted by adding 200µL Buffer AE to the centre of the spin column membrane. This was left to incubate for 1 minute at room temperature, then centrifuged for 1 minute at 6,164g. This elution step was repeated using the eluate in order to increase concentration of the DNA yield.

5.2.5.2 Polymerase chain reaction for SRY

A cocktail of reaction mix was prepared, consisting of 10µL of Roche LightCycler 480 Probes Master (Roche, Burgess Hill, UK), 2µL each of forward and reverse primers, 2µLDNA extract and 4µL of diethylpyrocarbonate (DEPC) treated water, to make up 20µL total volume in a PCR tube. The PCR was conducted using a Techne TC-512 Thermocycler (Techne, Cambridge, UK) at the following settings: initial denaturation at 94°C for 5 minutes, followed by 40 amplification cycles at 94°C for 30 seconds, annealing at 55°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 5 minutes. A total of 5µL of PCR product was used in gel electrophoresis, and 10µL was sent for direct Sanger sequencing.

5.2.6 Quantitative reverse transcription PCR standard curve and efficiency

The process of cDNA synthesis was as described in Chapter 2, Section 2.6, using the the qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, Maryland, USA). A total of three peri-renal AT cDNA samples were utilised for each sex and age group for qRT-PCR. In this study, age

groups were classified as adults and juveniles, where adults ranged between eight and 32 years old, and juveniles were from one to 3.5 years of age (Table 5.2). Age classification was according to a field key established by Varma and colleagues (2012), where elephants below five years old were classified as juveniles, five to fifteen years old as sub-adults, and above fifteen years old as adults. However, due to limitation of samples from older males, samples from two eight-year old males were placed in the adult male group to facilitate comparison.

qRT-PCR was conducted using the LightCycler® II Real-Time PCR system (Roche, Mannheim, Germany). A 96-well microtitre plate (LightCycler® 480 Multiwell Plate 96) was used for qRT-PCR with 80 - 140ng (2µL) of template cDNA in 15µL reaction volumes. The reaction mix consisted of 7.5µL PerfeCTa SYBR Green FastMix (Quanta Bioscience Inc. California, USA), a reaction cocktail composed of buffer, stabilizers, AccuFast™ Taq DNA polymerase (which contains a proprietary mixture of monoclonal antibodies that bind to the polymerase and keep it inactive prior to the initial PCR denaturation step), 2µL each of forward and reverse primers, and 1.5µL of RNase-free water. Finally 2µL of cDNA template was added to each well with reaction mix, while RNase free water was added to control wells, and the multiwell plate was sealed with LightCycler ® 480 Sealing Foil. The multiwell plate was placed in a standard microplate rotor centrifuge (Heraeus Labofuge 400, Thermo Scientific, Portsmouth, USA) and centrifuged for 2 minutes at 1,512g to collect the reaction mix and samples at the bottom of the wells.

PCR amplifications were performed with an initial 5 minutes denaturation at 94°C, followed by 44 cycles of 10 seconds at 95°C, and 30 seconds at 60°C. The initial qRT-PCR run incorporated 2 points of cycle threshold (Ct) acquisition after 10 seconds at 72°C and 80°C. Melting curve analysis from the first run revealed that melting curves occurred above 72°C but below 80°C, therefore it was decided to record fluorescence intensity at 80°C as primer-dimer was denatured at this temperature.

A melting curve run followed PCR amplification, in order to confirm that only one amplicon for each gene of interest was amplified during PCR, thus only one melting peak should appear in the melting curve analysis. Multiple melting peaks would have occurred if multiple fragments of cDNA, primer-dimers or other non-specific products were amplified. The reaction mixture was heated to 95°C, and fluorescence was continuously monitored as the temperature fell to 62°C.

A standard curve was generated for each primer pair, to calculate the amplification efficiency of qRT-PCR reaction based on the slope of the standard curve. The cDNA template was diluted in 1/5, 1/10, 1/20, 1/30, and 1/40 from the cDNA produced from reverse transcription of extracted RNA. The Ct values from qRT-PCR were plotted against their corresponding log dilution value, then the slope and coefficient of determination (r^2) was determined using Microsoft Excel chart tools. Efficiency of qRT-PCR was calculated by entering the slope gradient value of the standard curve into the online calculator on the Thermo Scientific Web Tools page (https://www.lifetechnologies.com/uk/en/home/brands/thermo-scientific/

molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html). The efficiency value was then used to calibrate the Ct value generated through qRT-PCR.

Table 5.2. List of samples for qRT-PCR of peri-renal AT from African elephants. Three adult and three juvenile samples were used for each sex. Adult age was estimated to be 8 years and above.

Sample ID	cDNA ID	Sex	Age/years	Age group
svc11-01	E-PF1C	F	25	Adult
svc11-02	E-PF2C	F	27	Adult
svc11-03	E-PF3C	F	28	Adult
svc11-23	E-PF23C	F	2.5	Juvenile
svc11-9	E-PF9C	F	2.7	Juvenile
svc11-8	E-PF8C	F	3	Juvenile
svc11-05	E-PF5C	M	8	Adult
svc11-18	E-PF18C	M	8	Adult
svc11-14	E-PF14C	M	16	Adult
svc11-10	E-PF10C	M	1	Juvenile
svc11-22	E-PF22C	M	3	Juvenile
svc11-20	E-PF20C	M	3.5	Juvenile

5.2.7 PCR product clean-up

QIAquick® PCR Purification Kit (Qiagen, Crawley West Sussex, UK) was used to clean PCR product from reagents used in amplification reactions. PCR products from the same primer pair were aspirated from the 96-well microtitre plate and pooled in a 1.5µL microcentrifuge tube. Five volumes of Buffer PB were added to one volume of the PCR reaction and mixed thoroughly by inverting the microcentrifuge tube. The binding buffers provided high concentrations of chaotropic salt, to optimise adsorption of cDNA to the QIAquick column membrane.

Prepared sample was applied to the QIAquick column and centrifuged for 60 seconds at 16,278g at room temperature to bind cDNA to the column membrane, while primers, reagents used during PCR, and salts flowed through the membrane into the collection tube and were discarded. The QIAquick column was placed into the same collection tube, and 75µL Buffer PE was added to the QIAquick column, and centrifuged for 60 seconds to wash out salts contained in Buffer PB. Flow-through was again discarded and the QIAquick column placed into the same collection tube. The QIAquick column was centrifuged once more in the collection tube for 1 minute to remove residual wash buffer, then transferred to a clean 1.5mL microcentrifuge tube. To elute cDNA, 30µl of elution buffer (EB) was added to the centre of the QIAquick membrane, left to stand for 1 minute, and then centrifuged for 1 minute.

5.2.8 Sanger sequencing

A total of 10µL of PCR products and 50µL of forward primer were sent for direct Sanger sequencing to confirm the identity of all PCR products (Source Bioscience, Nottingham, UK). Sequencing data was received in ABI format and viewed using Chromas Lite software (http://technelysium.com.au/?page_id=13). The nucleotide sequences were checked and any unknown nucleotide (N) was entered manually, and the sequence was saved and then exported in FASTA format. The FASTA file was uploaded onto the standard nucleotide BLAST page (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=Bla

stSearch&LINK_LOC=blasthome) and this generated an array of sequences in multiple species that were annotated in the database. The search result identified the species, genes, as well as percentage of sequence match between primers and genes in the database. This verified that designed primers were annealed specifically to the gene of interest. Further confirmation was obtained when the FASTA file was searched against the elephant genome through BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat?command=start). The cDNA and genomic sequences were displayed. Bases in blue indicated the primer sequence, and these was used to identify the exon-intron boundary on the sequencing data.

Nucleotide sequences viewed using Chromas Lite software were also used to identify the position of the exon-intron boundary in the sequence. This was used to further validate the primers designed to detect genes of interest in this study.

5.2.9 Gel electrophoresis

The PCR products were run on gel electrophoresis to validate the size of amplicon produced during qRT-PCR. A 1.5% agarose gel was prepared, made of 0.75g agarose gel powder (Fisher Scientific, Loughborough, UK), 50mL 1% TAE solution, and 3µL of Nancy-520 dye (Sigma-Aldrich, Dorset, UK). The gel form was placed in the gel chamber (Thermo Scientific, Portsmouth, USA) filled with 1x TAE solution, diluted from 50x TAE that was made up of 242g Tris Base with MW=121.1 (Sigma-Aldrich, Dorset, UK), 57.1mL Glacial Acetic Acid (Sigma-Aldrich, Dorset, UK), and 100mL 0.5 M EDTA (Fisher Scientific,

Loughborough, UK). A total of 6µL ϕX174 DNA-HaeIII Digest ladder (NEB, Ipswich, USA) was loaded into the first well of the gel, followed by 5µL of cleaned PCR products and 3µL of Orange G (Sigma-Aldrich, Dorset, UK). Electrophoresis was run for approximately 40 minutes, at 120volts and 400mAs. The gel was read using ImageQuant 300 (GE Healthcare Limited, Buckinghamshire, UK) under UV filters, and the image was recorded.

5.3 Results

5.3.1 Transcriptomic analysis

5.3.1.1 Statistics and read annotations

Paired sequence reads were generated using Illumina Hi-Seq platform for RNA-seq analysis. The breakdown of raw reads obtained, read numbers after quality control and mapping processes are shown in Table 5.3 and Figure 5.1. Both female samples were sequenced twice, while the male sample only sequenced once. The female samples were sequenced twice to generate sufficient reads as specified in the original contract with Edinburgh Genomics. Raw reads generated from both sequences were pooled and normalised, thus higher read numbers in Female1 and Female2 would not result in a greater mRNA expression level compared to Male1 (Figure 5.1). A total of 23,245 genes were annotated to the *Loxodonta africana* genome in the Ensembl database from the mapped reads, and these were used for subsequent analyses. Over 80% of raw reads were successfully mapped to the reference

genome, and the highest percentage of reads not usable was 18.61% in Female1, and lowest was 11.59% in Male1 (Figure 5.2).

Table 5.3. Breakdown of RNA-seq reads for adipose samples of female and male elephants. Numbers of raw reads generated from RNA-seq are shown as total reads. QC reads are sequence reads kept after QC, and the ones that did not pass the QC processes are the discarded reads. Sequence reads that were confidently mapped to reference genome are presented as mapped reads, leaving the ones that were not mapped as unmapped reads.

ID	Age	Total reads	QC reads	Mapped reads	Unmapped reads	Discarded reads
Female1	25 y	63,041,074 (100%)	60,966,414 (96.70%)	51,310,318 (81.39%)	9,656,096 (15.32%)	2,074,660 (3.29%)
Female2	32 y	83,963,786 (100%)	81,437,446 (96.99%)	71,796,269 (85.51%)	9,641,177 (11.48%)	2,526,340 (3.01%)
Male1	16 y	56,783,624 (100%)	55,005,556 (96.86%)	50,204,080 (88.41%)	4,801,476 (8.46%)	1,778,068 (3.13%)

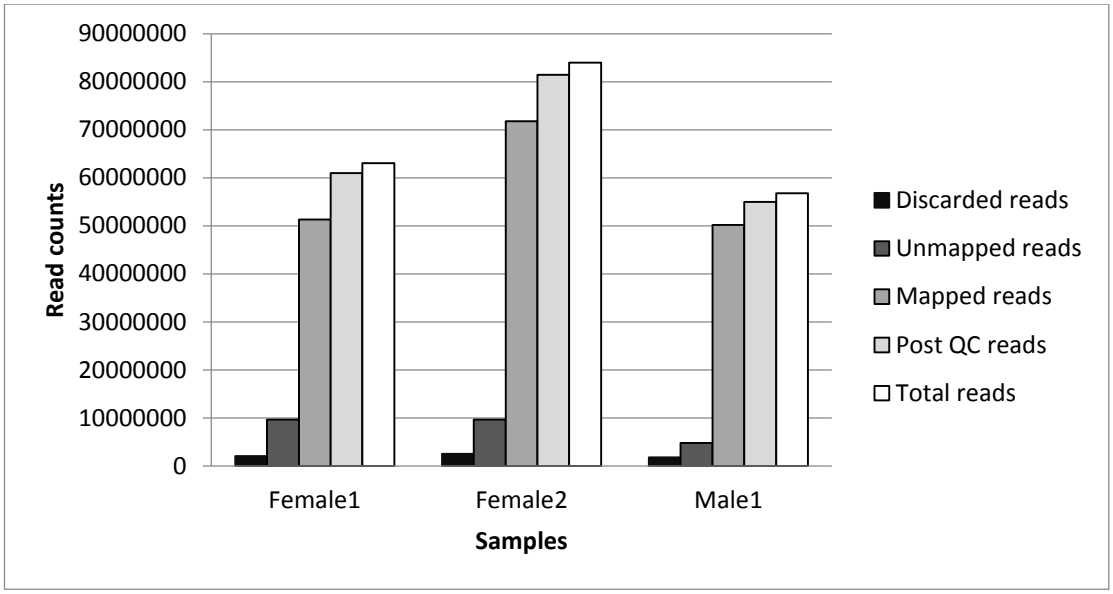


Figure 5.1. RNA-seq reads dispersions of AT from female and male African elephants by count numbers. Total reads refer to the number of raw reads generated from RNA-seq. Post QC reads are sequence reads that passed QC criteria, and the discarded reads are the ones that failed to pass QC. Mapped reads are sequence reads confidently mapped to reference genome, while the ones that were not mapped are presented here as unmapped reads.

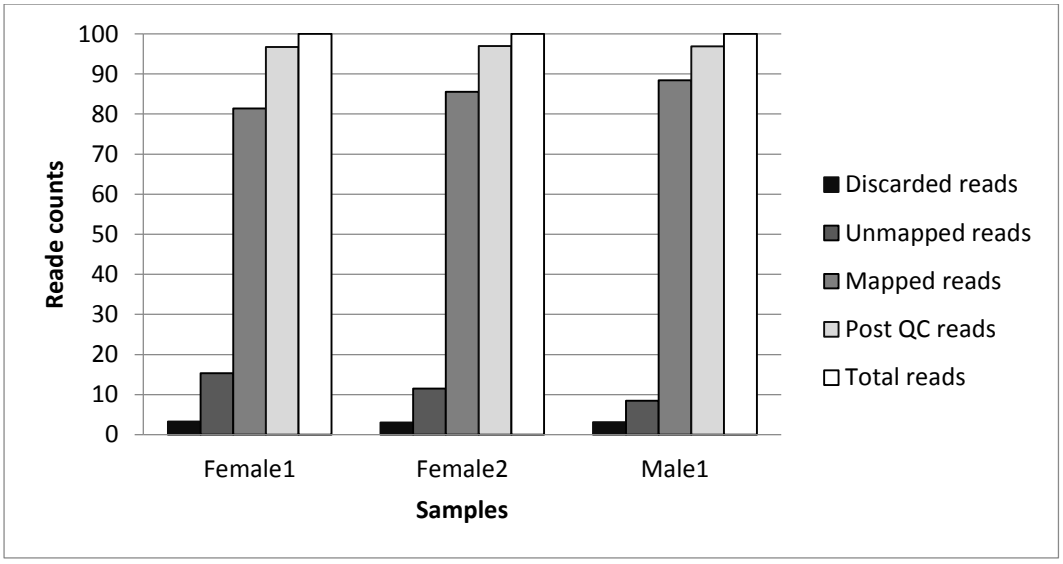


Figure 5.2. RNA-seq reads dispersions of AT from female and male African elephants by percentage of count numbers. This graph shows the breakdown of sequence reads in percentages corresponding to count numbers presented in Figure 5.1.

5.3.1.2 Differentially expressed genes

5.3.1.2.1 Comparison between sexes

A total of 28 genes were significantly expressed at a higher level in the male compared to the female elephants (Table 5.4), whereas only 17 genes had lower expression (Table 5.5). A number of the 28 transcripts annotated to known genes were found to be related to muscle development or muscular functions such as contraction and motility: *CNN1*, *TCAP*, *ACTG2, DES*, *ACTA1*, *TPM2* (Lehtonen et al., 2012, Citirak et al., 2014, Francis et al., 2014, Lu et al., 2014, Koutakis et al., 2015, Zukosky et al., 2015) according to information deposited in NCBI Reference Sequence Database, RefSeq (http://www.ncbi.nlm.nih.gov/refseq/). Other genes were reported to be involved in immune responses: *IL17B* and *LTC4S* (Zhang et al., 2012, Kouri et al., 2014); ion channel: *KCNH2* (Jones et al., 2014); and energy homeostasis: *CKB* (Lin et al., 2013). These were suggestive of enhanced muscular tissue development in the male compared to females.

On the other hand, genes with higher expression in the females are involved in multiple functions and activities, such as cell motility and polarity, blood vessel formation, organ development and wound healing, hormone excretion, water and ion influx, and RNA interference (Table 5.5). The genes *LEP* and *ABCA1* were of interest as these are related to energy homeostasis and cellular lipid movement (Cohen and Friedman, 2004, de Haan et al., 2014), and these differences may indicate a functional difference in energy expenditure and storage between the sexes. Distribution of DEG between the

sexes are shown in Figures 5.3, 5.4 and 5.5. Filtering the gene lists for genes with a FC $\geq$ 2, narrowed down the pool of genes of interest.

Table 5.4. A total of 28 genes with lower mRNA expression in female African elephants compared to the male ordered by the amount of fold change.

Ensembl ID	Log FC	Official symbol	Gene description
ENSLAFG00000001531	4.33759	*CNN1*	Calponin 1, basic, smooth muscle
ENSLAFG00000000281	4.13861	*TCAP*	Titin-cap
ENSLAFG00000010951	3.74487	*LXN*	Latexin
ENSLAFG00000012628	3.52518	*ACTG2*	Actin, γ2, smooth muscle, enteric
ENSLAFG00000021716	3.40328	*IL17B*	Interleukin 17B
ENSLAFG00000015024	3.25688	*ASB2*	Ankyrin repeat and SOCS box containing 2
ENSLAFG00000003073	3.20070	*KCNH2*	Potassium voltage-gated channel, subfamily H (eag-related), member 2
ENSLAFG00000009073	3.19989	*DES*	Desmin
ENSLAFG00000028487	3.15811	*LOC100661895*	Uncharacterized protein
ENSLAFG00000011927	3.13083	*COX6A2*	Cytochrome c oxidase subunit VIa polypeptide 2
ENSLAFG00000009429	3.10534	*MAP3K15*	Mitogen-activated protein kinase 15
ENSLAFG00000018170	3.09191	*HSPB7*	Heat shock 27kDa protein family, member 7 (cardiovascular)
ENSLAFG00000006306	3.03789	*ACTA1*	Actin, α1, skeletal muscle
ENSLAFG00000014870	3.02386	*MYH11*	Myosin, heavy chain 11, smooth muscle
ENSLAFG00000000496	2.97038	*TPM2*	Tropomyosin 2 (β)
ENSLAFG00000029939	2.96865	*ARHGAP40*	Rho GTPase activating protein 40
ENSLAFG00000011696	2.95104	*RERGL*	RERG/RAS-like
ENSLAFG00000031986	2.69851	-	Uncharacterized protein
ENSLAFG00000031957	2.63606	-	Uncharacterized protein
ENSLAFG00000000825	2.62955	*COX7A1*	Cytochrome c oxidase subunit VIIa polypeptide 1 (muscle)
ENSLAFG00000031612	2.60793	*CKB*	Creatine kinase, brain
ENSLAFG00000032491	2.54626	-	Novel gene
ENSLAFG00000031008	2.51925	*U3*	Small nucleolar RNA U3
ENSLAFG00000016972	2.46320	*MMP17*	Matrix metallopeptidase 17 (membrane-inserted)
ENSLAFG00000020490	2.44996	*LTC4S*	Leukotriene C4 synthase
ENSLAFG00000000079	2.44123	*ARHGAP22*	Rho GTPase activating protein 22
ENSLAFG00000004129	2.40823	*COL4A4*	Collagen, type IV, alpha 4
ENSLAFG00000030581	2.26110	*RGS2*	regulator of G-protein signaling 2, 24kDa

Table 5.5. A total of 17 genes with higher mRNA expression in female African elephants compared to male ordered by the amount of fold change.

Ensembl ID	Log FC	Official symbol	Gene description
ENSLAFG00000033167	-5.74097	-	Novel gene
ENSLAFG00000003179	-5.28765	*KRT18*	Keratin 18
ENSLAFG00000010714	-4.42881	*LEP*	Leptin
ENSLAFG00000015516	-4.21850	*ABCA1*	ATP-binding cassette, sub-family A, member 1
ENSLAFG00000029077	-4.20581	-	Uncharacterized protein
ENSLAFG00000011961	-4.14239	*NOTCH2*	Notch 2
ENSLAFG00000030249	-3.91329	*KIAA0754*	KIAA0754
ENSLAFG00000026357	-3.64274	*AHNAK2*	AHNAK nucleoprotein 2
ENSLAFG00000010835	-3.60371	*AGO1*	Argonaute RISC catalytic component 1
ENSLAFG00000011076	-3.59703	*ADAMTSL1*	ADAMTS-like 1
ENSLAFG00000013359	-3.58303	*SYT1*	Synaptotagmin I
ENSLAFG00000015945	-3.31147	*MYH14*	Myosin, heavy chain 14, non-muscle
ENSLAFG00000008128	-3.17714	*SCUBE1*	Signal peptide, CUB domain, EGF-like 1
ENSLAFG00000016525	-3.13434	*CYBRD1*	Cytochrome b reductase 1
ENSLAFG00000008818	-3.12394	*THBS4*	Thrombospondin 4
ENSLAFG00000010795	-3.11064	*PDGFRA*	Platelet-derived growth factor receptor, α polypeptide
ENSLAFG00000003360	-2.80311	*VIPR2*	Vasoactive intestinal peptide receptor 2

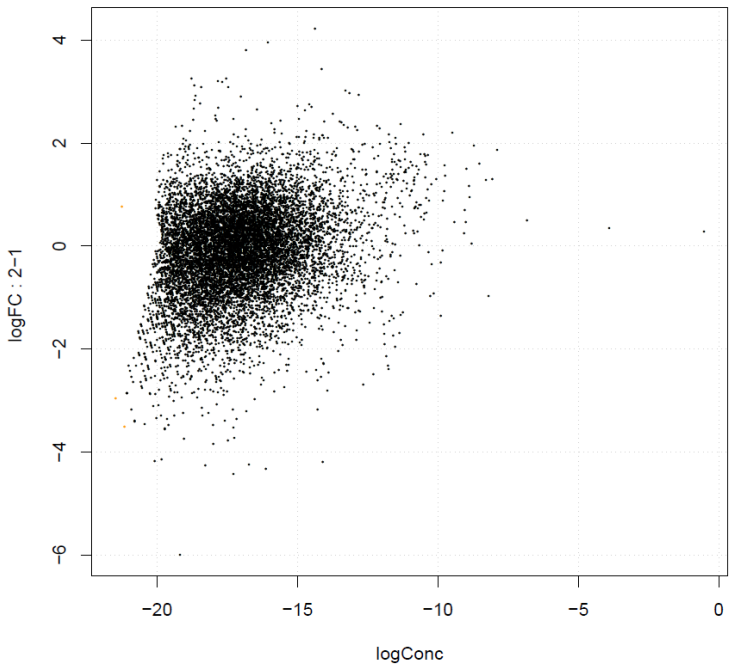


Figure 5.3. Smear plot comparing log FC and –log average gene expression counts on differential gene expression of AT in female versus male elephants. This plot shows distribution of annotated genes.

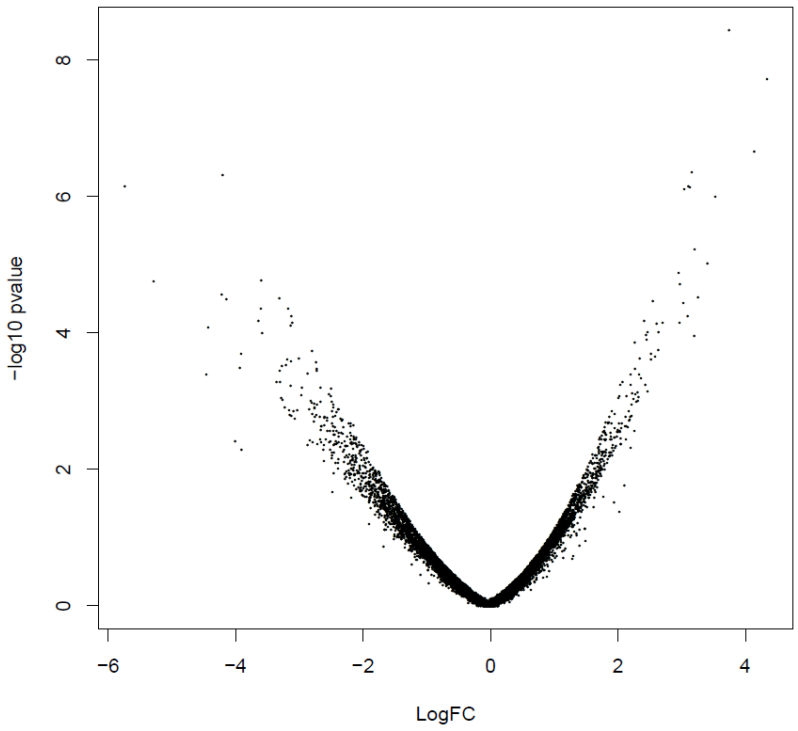


Figure 5.4. Volcano plot comparing –log10 p-value and log FC on differential gene expression of AT in female versus male elephants. Volcano plot with DE genes of interest with FC $\geq$ 2 in the upper left and right regions.

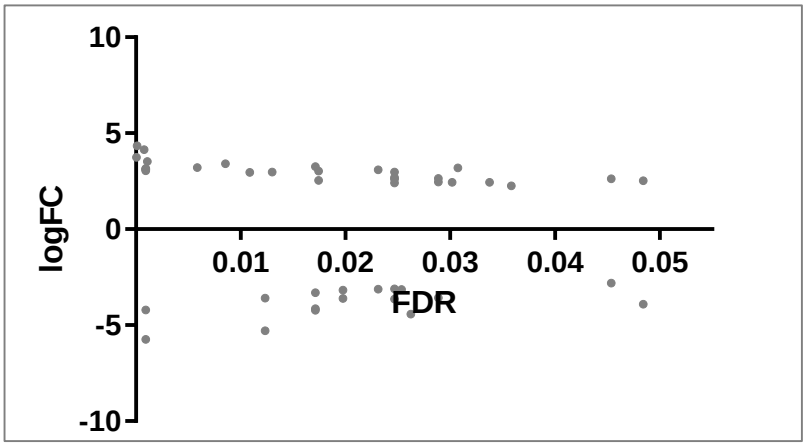


Figure 5.5. Differential gene distribution by log FC versus FDR on gene expressions of AT in female versus male elephants.

5.3.1.2.2 Comparison between females

The female samples were compared with one another as RNA-seq results showed that these replicates were more different from one another than may be expected. A total of 2,809 genes were expressed in a higher level in the older female (32 years) compared to the younger female (25 years), while 2,691 genes had significantly lower expression in the older female compared to the younger female (Appendix 5). Filtering the gene lists for genes with FC $\geq$ 2 and FDR < 0.05, narrowed down the pool of genes of interest (Figures 5.6, 5.7, and 5.8).

Over a third of the top 25 genes with lower mRNA expression in the younger females were functionally involved in lipid metabolism and biosynthesis: *DGAT2*, *THRSP*, *SCD*, *LEP*, *DB1*, *ELOVL6*, and *ME1* (Cohen and Friedman, 2004, Strable and Ntambi, 2010, Suzuki et al., 2005, Ortega et al., 2010, Matsuzaka and Shimano, 2009, Burgi et al., 1999, Al-Dwairi et al., 2012) based on information obtained from the NCBI Reference Sequence Database, RefSeq (http://www.ncbi.nlm.nih.gov/refseq/). This is suggestive of higher adiposity and lipogenesis in the older female compared to the younger individual. Other genes were related to various functions and activities such as immune response, organ development, electron transfer, DNA repair, replication and recombination, also RNA processing and degradation (Table 5.6).

The genes with higher mRNA expression in the younger female are involved in diverse physiological functions, including the immune response: *NFKBIZ*, *SOCS3*, *SELE*, *IER3*, and *NFAT5* (Chapman et al., 2010, Ishimoto et al., 2011,

Konnecke et al., 2014, Zafra et al., 2015), cell proliferation, differentiation and transformation: *FOS*, *FOSB*, *ID1*, *JUN*, *JUNB*, *KLF4,* and *SNAI1* (Oliveira-Ferrer et al., 2014, Satyanarayana et al., 2012, Luo et al., 2014, Jiang et al., 2014, Lee et al., 2014), haematopoiesis and hypertension: *DLL1*, and *SGK1* (Lang et al., 2010, Marcelo et al., 2013). *FBN3* has been reported to have a role in the pathogenesis of polycystic ovary syndrome (PCOS) in human patients (Jordan et al., 2010). *OSR2* was shown to supress tooth development after eruption of permanent teeth in mammals (Jheon et al., 2013). Finding expression of *OSR2* within the list of genes with high FC may be due to the fact that the elephant tusk is a modified incisor that continues to grow throughout the animal's life (Table 5.7).

Table 5.6. Top 25 genes with lower mRNA expression in 25 years old female African elephant compared to 32 year old female ordered by the amount of fold change.

Ensembl ID	Log FC	Official symbol	Gene description
ENSLAFG00000006514	5.18305	*SPP1*	Secreted phosphoprotein 1
ENSLAFG00000003799	3.66268	*DGAT2*	Diacylglycerol O-acyltransferase 2
ENSLAFG00000012759	3.56009	*ECHDC1*	Enoyl CoA hydratase domain containing 1
ENSLAFG00000009790	3.55061	*TNFAIP6*	Tumour necrosis factor, α-induced protein 6
ENSLAFG00000022714	3.31822	*THRSP*	Thyroid hormone responsive
ENSLAFG00000017790	3.16670	*SCD*	Stearoyl-CoA desaturase (Δ-9-desaturase)
ENSLAFG00000010664	3.15192	*FBP2*	Fructose-1,6-bisphosphatase 2
ENSLAFG00000009787	2.99543	*MMRN1*	Multimerin 1
ENSLAFG00000011486	2.92353	*STMN2*	Stathmin 2
ENSLAFG00000016209	2.90826	*TYR*	Tyrosinase
ENSLAFG00000010714	2.71302	*LEP*	Leptin
ENSLAFG00000022206	2.67559	*FABP3*	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
ENSLAFG00000005002	2.61890	*DBI*	Diazepam binding inhibitor (GABA receptor modulator, acyl-CoA binding protein)
ENSLAFG00000015005	2.61088	*S100A5*	S100 calcium binding protein A5
ENSLAFG00000012451	2.54508	*REXO2*	RNA exonuclease 2
ENSLAFG00000003267	2.51137	*NEXN*	Nexilin (F actin binding protein)
ENSLAFG00000010885	2.48138	*TAGLN3*	Transgelin 3
ENSLAFG00000021532	2.37837	*ELOVL6*	ELOVL fatty acid elongase 6
ENSLAFG00000006155	2.33986	*ACSM3*	Acyl-CoA synthetase medium-chain family member 3
ENSLAFG00000014935	2.29584	*S100B*	S100 calcium binding protein B
ENSLAFG00000032759	2.28994	*AGT*	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
ENSLAFG00000011855	2.20651	*NDUFA4*	NADH dehydrogenase (ubiquinone) 1α subcomplex, 4
ENSLAFG00000002078	2.19588	*HRSP12*	Heat-responsive protein 12
ENSLAFG00000009874	2.16364	*FAM195A*	Family with sequence similarity 195, member A
ENSLAFG00000005140	2.15508	*ME1*	Malic enzyme 1, NADP(+)-dependent, cytosolic

Table 5.7. Top 25 genes with higher mRNA expression in 25 year old female African elephant compared to 32 year old female ordered by the amount of FC ordered by the amount of fold change.

Ensembl ID	Log FC	Official symbol	Gene description
ENSLAFG00000008834	-6.05516	*FOSB*	FBJ murine osteosarcoma viral oncogene homolog B
ENSLAFG00000001888	-4.37042	*NFKBIZ*	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ζ
ENSLAFG00000007199	-4.15594	*CETP*	Cholesteryl ester transfer protein, plasma
ENSLAFG00000025968	-3.87733	*SOCS3*	Suppressor of cytokine signalling 3
ENSLAFG00000029435	-3.49136	*FOS*	FBJ murine osteosarcoma viral oncogene homolog
ENSLAFG00000007166	-3.29389	*SELE*	Selectin E
ENSLAFG00000027353	-2.97691	*BTBD19*	BTB (POZ) domain containing 19
ENSLAFG00000003820	-2.93275	*SEMA5A*	Sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A
ENSLAFG00000001043	-2.92826	*DLL1*	Delta-like 1 (Drosophila)
ENSLAFG00000012698	-2.80305	*SGK1*	Serum/glucocorticoid regulated kinase 1
ENSLAFG00000017323	-2.78410	*IER3*	Immediate early response 3
ENSLAFG00000015516	-2.69126	*ABCA1*	ATP-binding cassette, sub-family A, member 1
ENSLAFG00000008944	-2.65156	*GLI3*	GLI family zinc finger 3
ENSLAFG00000012900	-2.61205	*JUN*	Jun proto-oncogene
ENSLAFG00000009900	-2.56919	*ID1*	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein
ENSLAFG00000011286	-2.55724	*ZNF618*	Zinc finger protein 618
ENSLAFG00000030249	-2.51481	*KIAA0754*	KIAA0754
ENSLAFG00000030265	-2.50933	*SNAI1*	Snail family zinc finger 1
ENSLAFG00000014805	-2.50868	*KMT2A*	Lysine (K)-specific methyltransferase 2A
ENSLAFG00000008530	-2.50356	*JUNB*	Jun B proto-oncogene
ENSLAFG00000009420	-2.49090	*OSR2*	Odd-skipped related transciption factor 2
ENSLAFG00000029310	-2.47680	*FBN3*	Fibrillin 3
ENSLAFG00000006799	-2.45619	*TMEM132E*	Transmembrane protein 132E
ENSLAFG00000000421	-2.45204	*KLF4*	Kruppel-like factor 4 (gut)
ENSLAFG00000008964	-2.43940	*NFAT5*	Nuclear factor of activated T-cells 5, tonicity-responsive

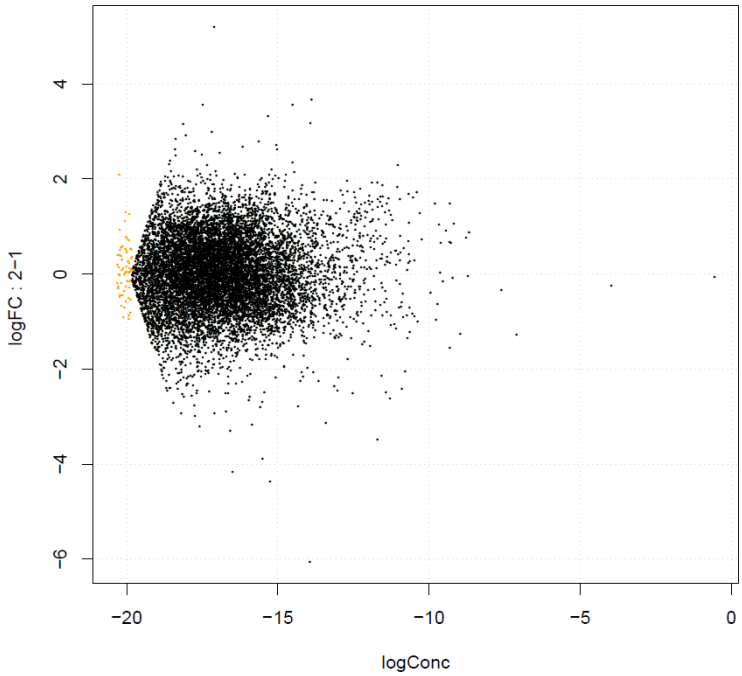


Figure 5.6. Smear plot comparing log FC and –log average gene expression counts on differential gene expression of AT in female elephants, 25 versus 32 year old. This plot shows distribution of annotated genes.

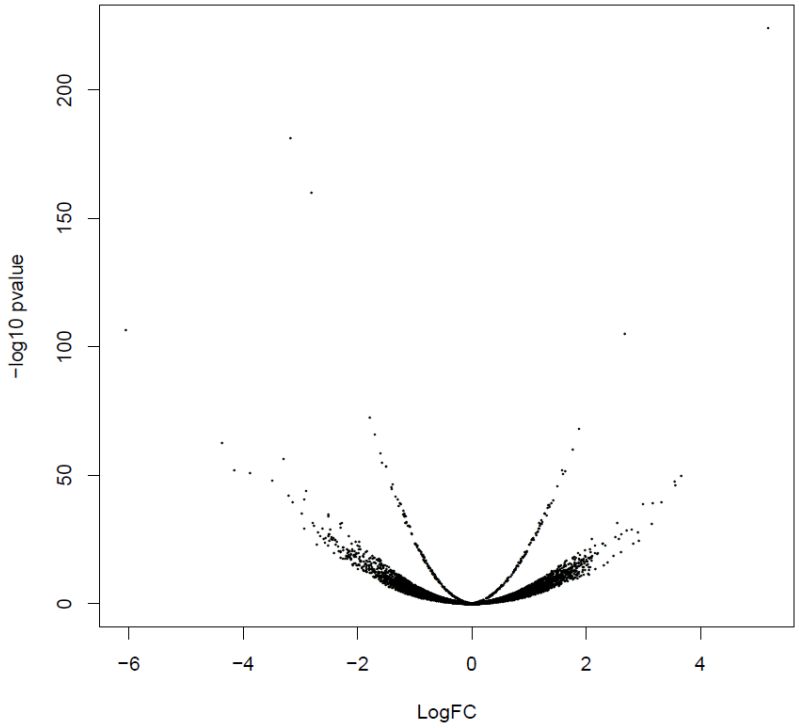


Figure 5.7. Volcano plot comparing –log10 p-value and log FC on differential gene expression of AT in female elephants, 25 year old versus 32 year old. Volcano plot with DE genes of interest with FC $\geq$ 2 in the upper left and right regions.

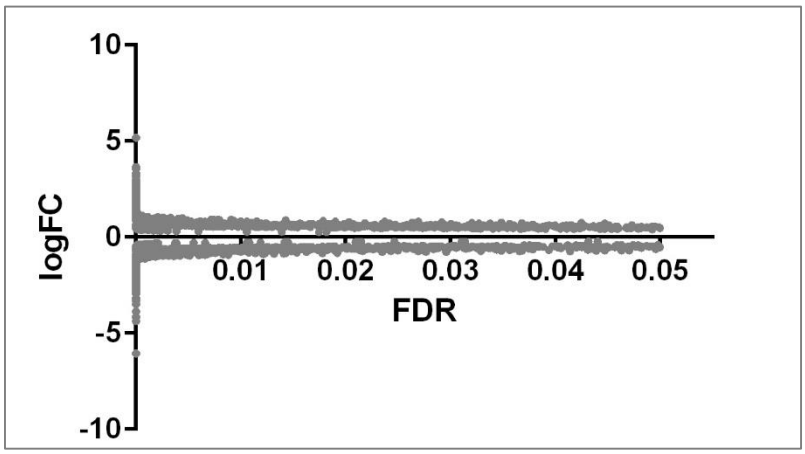


Figure 5.8. Differential gene distribution by log FC versus FDR on differential gene expression of AT in female elephants, 25 versus 32 years old.

5.3.2 Hierarchical cluster analysis between female and male African elephants

Hierarchical clustering of genes expressed in peri-renal AT of female and male elephants were generated from the total of 23,245 genes identified through RNA-seq. The numbers of genes were filtered down to 317 with all 3 samples with read counts $\geq$ 50, and FC $\geq$ 2. It appeared that mRNA expression of Female2 was closer to that of Male1 than to that of Female1 (Figure 5.9). This was also observed in Figures 6.2 and 6.3 illustrating cross species comparison of AT transcriptomes. Thus, it was reasonable to consider that the sex of the animals from which these samples were taken may have been incorrectly assigned in the field during sample collection. Therefore, it was important to confirm the sex of the animal from which each sample was taken before further analysis was performed (Figure 5.11).

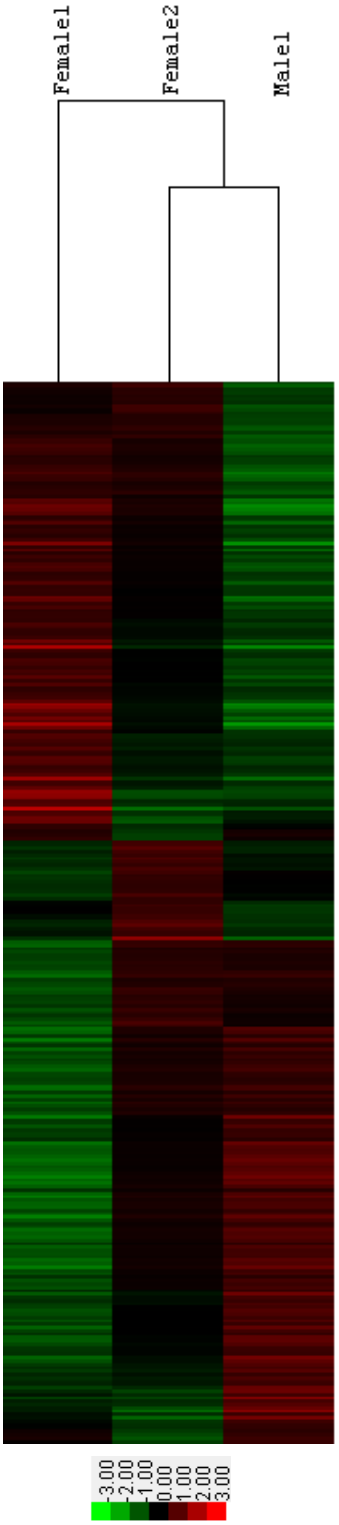


Figure 5.9. Hierarchical clustering of genes expressed in peri-renal AT of female and male elephants. From a total of 23245 genes identified through RNA-seq, numbers of genes were filtered down to 317 with all 3 samples with $\geq$ 50 read counts, and FC $\geq$ 2. This figure shows that mRNA expression of Female2 was closer to that of Male1. Female n = 2, Male n = 1.

5.3.3 Determination of the sex of the animals

Because the elephant reference genome had been sequenced from a female, an elephant-specific sex determining region Y (*SRY*) sequence could not be determined from the existing elephant sequence data. Therefore, *SRY* forward and reverse primers were designed using homologous genomic DNA sequences from common regions of the *SRY* sequences from humans and other domestic and wild animal species. The PCR products were then sent for direct Sanger sequencing and results were compared with known SRY sequences from several animal species. Figure 5.10 demonstrates that the nucleotide sequences of elephant *SRY* primers were generally conserved in most regions between species, thus verifying the likely validity of these primers for detecting this gene in the African elephant.

The PCR results for detecting *SRY* in male and female elephant peri-renal adipose samples confirmed that the Male1 adipose sample sent for NGS was truly male. As can be seen in Figure 5.11, the sample from Male1 harboured the *SRY* gene (white circle). This also confirmed that mRNA expression between the 2 female samples were truly between two animals of same sex, although they had a relatively high number of significant differentially expressed genes (DEG). However, as comparison between these 2 samples would not generate much insight besides highlighting individual differences, the focus was instead on comparison of gene expression differences between sexes.

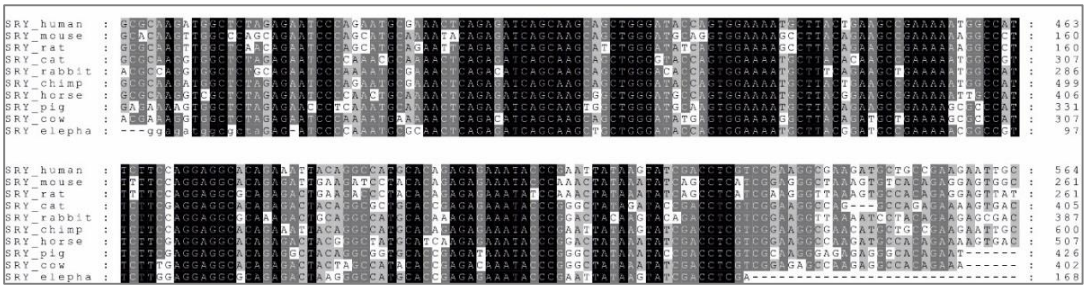


Figure 5.10. Comparison of nucleotide sequences of SRY primers in African elephants to human and other domestic and wildlife species. The elephant *SRY* forward and reverse primers were designed using common regions from human and other animals. This figure shows *SRY* nucleotide sequences were generally conserved in most regions between species, and nucleotide sequence of elephant SRY primers from Sanger sequencing was found closely matching the sequences from other species, thus confirming the validity of *SRY* primers designed for African elephant.

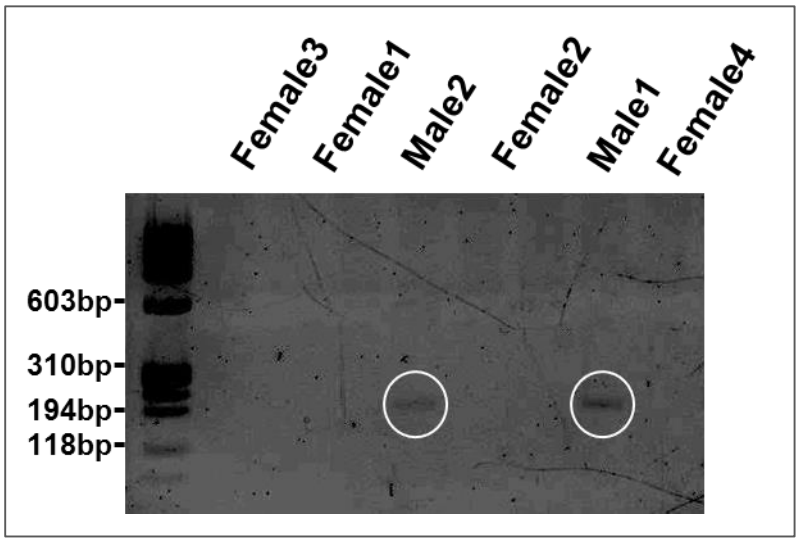


Figure 5.11. PCR of SRY in male and female elephant peri-renal adipose samples. Male1 sample sent for NGS was confirmed male, positively detected habouring the *SRY* gene (white circle). Female3, Female4 and Male2 (white circle) were additional adipose samples used for qRT-PCR validation.

5.3.4 Conventional PCR validation

Genes of interest were selected in accordance with their functions relating to adipogenesis, lipogenesis, or energy homeostasis, rather than their FC values (which, except for *LEP*, were < 2.0; see Table 5.9). To extend this analysis, juvenile samples were also analysed to determine the differences between adults and juveniles, as well as between sexes (Figures 5.14 to 5.20).

Before running qRT-PCR to validate DEG between sexes and age groups, the accuracy and specificity of the designed primers need to be validated. Therefore, PCR products from qRT-PCR performed to determine the amplification efficiency of the PCR reaction were sent for Sanger sequencing. The results were then submitted to BLAST search and matched to the respective genes annotated in the African elephant reference genome. Direct Sanger DNA sequencing confirmed the identity of the transcripts, which demonstrated >90% similarity to gene sequences in the NCBI depository (Table 5.8) and the presence of a conserved exon boundary was also demonstrated (Figure 5.12). The sizes of these PCR products were demonstrated to match the predicted sizes from the designed primers by gel electrophoresis (Figure 5.13). Matching of sizes between PCR products and primers further confirmed the validity of designed primers in detecting the corresponding transcribed genes.

An amino acid variance was discovered in the ligand-binding domain region of *PPARγ* sequences between human and African elephant, the human sequence contained aspartic acid, while the African elephant contained glutamic acid. However, preliminary analysis suggests that this difference

does not lead to a significant change in the molecular structure of the gene, and therefore is unlikely to cause substantial alterations in the molecular functions of this gene between the two species (Nigel P. Mongan, pers comm).

Table 5.8. Automated Sanger DNA sequencing results of the PCR products of selected DEG, and percent similarity to gene sequences in the African elephant reference genome.

Genes	Accession number	Similarity %
ELOVL fatty acid elongase 5 *(ELOVL5*)	XM_003404427.2	99
Fatty acid synthase (*FASN*)	XM_010596872.1	94
Leptin (*LEP*)	XM_003407251.2	99
Nuclear receptor subfamily 2, group F, member 2 (*NR2F2*)	XM_003413903.2	100
Peroxisome proliferator-activated receptor γ (*PPARγ*)	XM_003409970.2	97
Retinoid X receptor, α (*RXRα*)	XM_010602203.1	98
Stearoyl-CoA desaturase (Δ-9-desaturase) (*SCD*)	XM_003409176.2	99

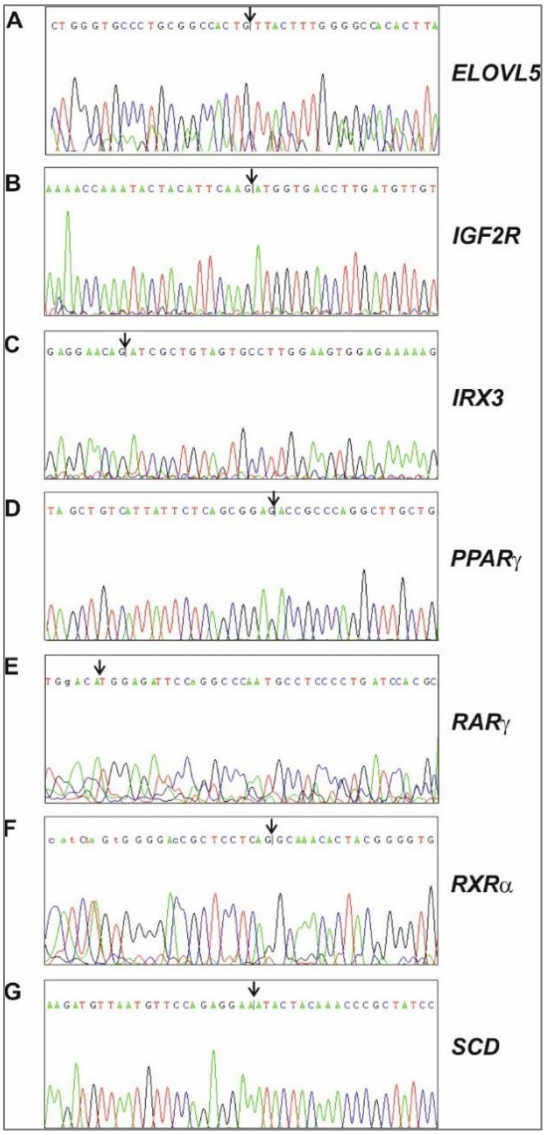


Figure 5.12. Nucleotide sequences from Sanger sequencing of PCR products of the genes of interest. The exon-intron junctions are indicated by an arrow (⇓).

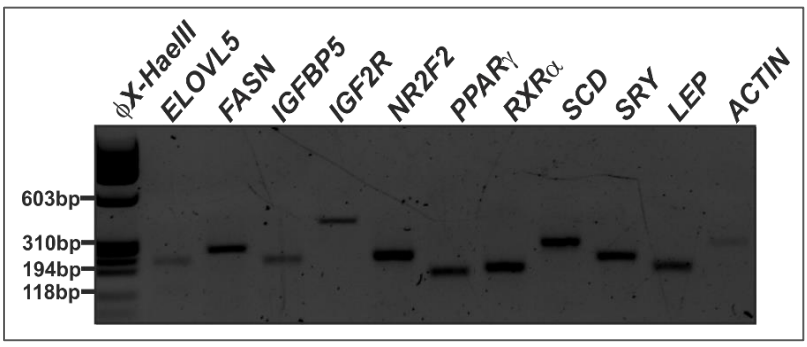


Figure 5.13. PCR validates the designed primers accurately match the nucleotide sequences of genes of interest in peri-renal AT of African elephants. The sizes of the PCR products also match the sizes predicted from the designed primers.

Table 5.9. Gene expression count values and FC between male and female African elephants through RNA-seq. F1 = Female1, F2 = Female2, M1 = Male1.

Genes	F1	F2	M1	Male vs Females Log FC (FDR ≤ 0.05)
ELOVL fatty acid elongase 5 *(ELOVL5*)	399	1017	241	-0.90
Fatty acid synthase (*FASN*)	5233	20013	10957	0.44
Leptin (*LEP*)	324	3963	50	-4.42
Nuclear receptor subfamily 2, group F, member 2 (*NR2F2*)	333	280	204	0.06
Peroxisome proliferator-activated receptor γ (*PPARγ*)	531	2714	1596	0.62
Retinoid X receptor, α (*RXRα*)	430	457	130	-1.11
Stearoyl-CoA desaturase (Δ-9-desaturase) (*SCD*)	607	7857	2117	-0.34

5.3.5 Quantitative reverse transcription PCR validation

Results from qRT-PCR comparing ELOVL fatty acid elongase 5 (*ELOVL5*) expression between adult females and males confirmed the results from RNA-seq. Both methods indicated there was not a significant difference in expression of this gene between adult males and females. However, qRT-PCR findings suggested it was significantly different between male and female juveniles. When comparing between the age groups, *ELOVL5* was expressed at a higher level in the adults compared to the juveniles (Figure 5.14).

By contrast, although RNA-seq results indicated that fatty acid synthase (*FASN*) expression was not significantly different between adults of both sexes, qRT-PCR results suggested that females of both age groups had lower expression than males. Furthermore, both female and male adults had greater expression than the juveniles of either sex (Figure 5.15). The qRT-PCR result

in adult females and males validated the significantly higher expression of leptin (*LEP*) in adult females suggested by the RNA-seq results, however there was not a significant difference in expression between male and female juveniles. The qRT-PCR results indicated that *LEP* expression was higher in female adults compared to female juveniles, but was conversely lower in adult males compared to male juveniles (Figure 5.16).

The qRT-PCR results confirmed the RNA-seq finding that expression of nuclear receptor subfamily 2, group F, member 2 (*NR2F2*) between adult females and males was not significantly different (Figure 5.17). However, previous report showed that NR2F2 mRNA was differentially expressed between sexes in African elephant (Nilsson et al., 2014). As the samples for microarray used in the previous study were from both juvenile and adults, in the current study, qRT-PCR on the adult and juveniles samples was carried out to determine if age affects the mRNA expression of *NR2F2*. Results from qRT-PCR suggested that the expression of this gene was significantly different between the male and female juveniles. Age also appeared to affect level of *NR2F2* expression, as it was lower in both female and male adults compared to juveniles (Figure 5.17).

RNA-seq results showed that peroxisome proliferator-activated receptor γ (*PPARγ*) mRNA was not differentially expressed between adult male and females. However, the individual *PPARγ* mRNA expression of Female1 and Female2 was significantly different compared to Male1 (Table 5.9). By contrast, qRT-PCR results comparing between adult females and males showed significantly higher *PPARγ* mRNA expression in the females. In the

juveniles, the expression of *PPARγ* was higher in the males compared to females. Gene expression in different age groups was also significantly different, *PPARγ* mRNA expression was higher in both female and male juveniles compared to the adults (Figure 5.18).

The qRT-PCR results indicated retinoid X receptor α (*RXRα*) mRNA expression was significantly higher in adult females compared to the males, in agreement with RNA-seq results. The juveniles similarly exhibited higher expression in the females than the males. Levels of *RXRα* mRNA expression were lower in both female and male adults compared to juveniles (Figure 5.19).

Similar to *PPARγ*, RNA-seq results shows that stearoyl-CoA desaturase (*SCD*) mRNA expression was not significantly different between the male and female adults, but when tested by qRT-PCR, females exhibited lower expression than males in both age groups. Additionally, *SCD* was less expressed in both female and male adults compared to juveniles, although this difference was not statistically significant in the males (Figure 5.20).

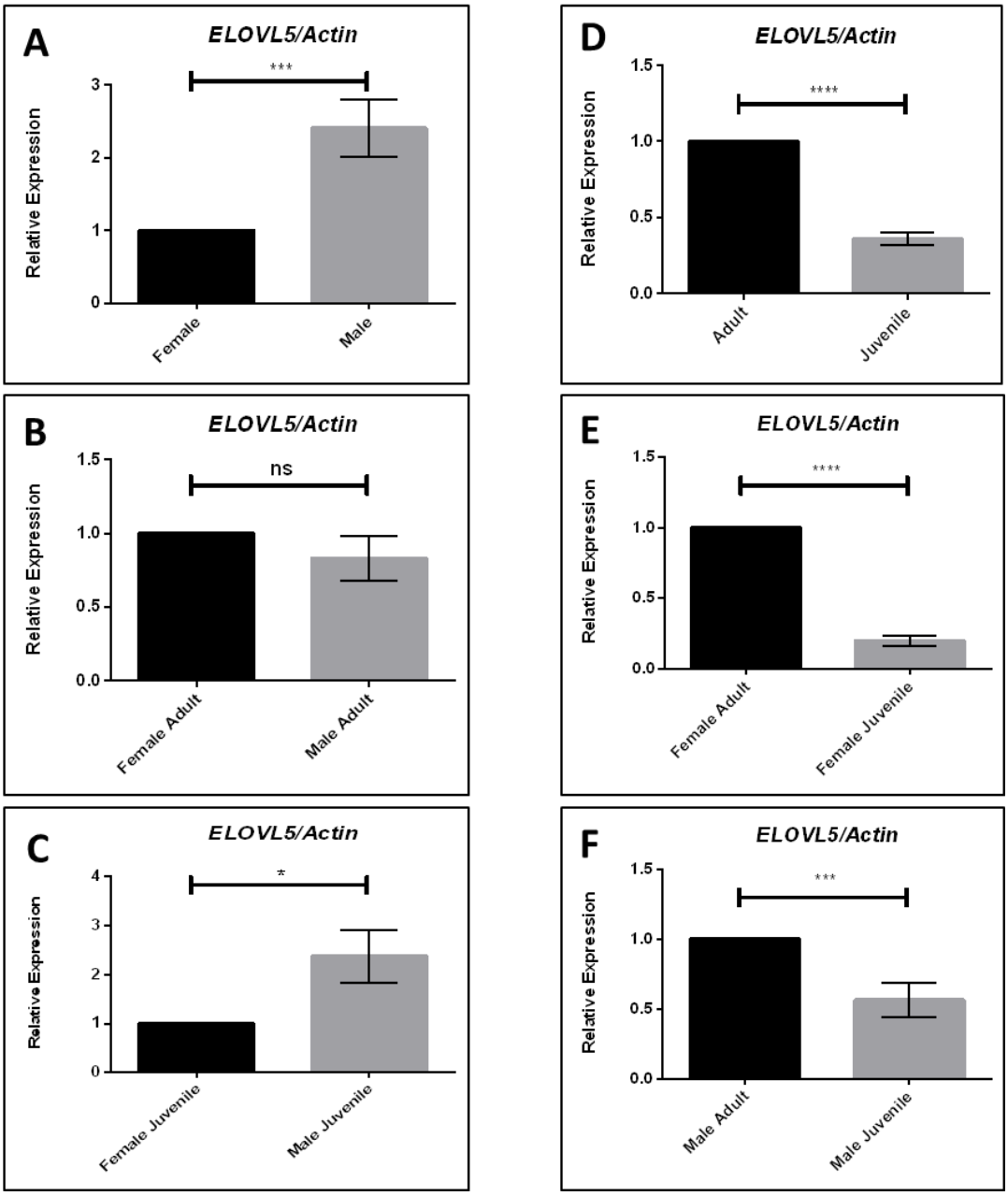


Figure 5.14. qRT-PCR validation of *ELOVL5* between peri-renal AT of females and males, adults and juveniles of African elephants. *ELOVL5* mRNA expression was significantly higher in the males compared to the females (A), this appeared to have an age influence as breakdown of the samples into different age groups showed significant differential expression was detected only in the juveniles (C). The differences between adult females and males was insignificant, and this is consistent with the data obtained from RNA-seq (B and Table 5.9). The mRNA expression of *ELOVL5* was significantly higher in the adults compared to juveniles (D), and this was observed in both the female and male groups (E and F). Mean values ± SEM. Level of significance: ns = non-significant, * = $p < 0.05$, *** = $p < 0.0005$, **** = $p < 0.0001$. (n=3 for each of age group and sexes).

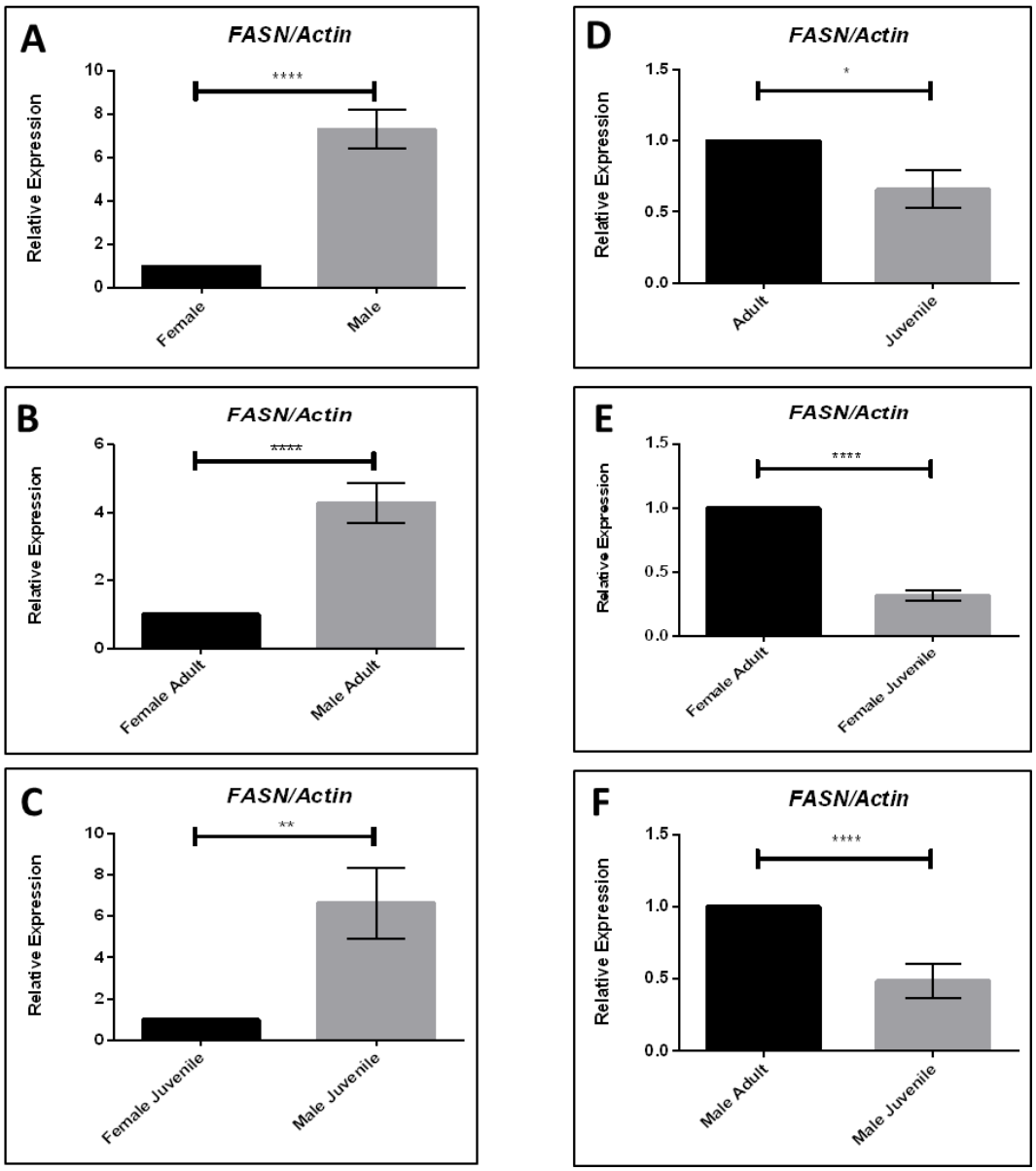


Figure 5.15. qRT-PCR validation of *FASN* between peri-renal AT of females and males, adults and juveniles of African elephants. mRNA expression of *FASN* was differentially expressed between sexes and age groups, it was consistently higher in the males compared to females (A, B, and C), and higher in the adults compared to juveniles (D, E, and F). However, the qRT-PCR results between adult females and males was not consistent with the data obtained from RNA-seq (Table 5.9). Mean values ± SEM. Level of significance: ns = non-significant, * = $p < 0.05$, ** = $p < 0.005$, **** = $p < 0.0001$. (n=3 for each of age group and sexes).

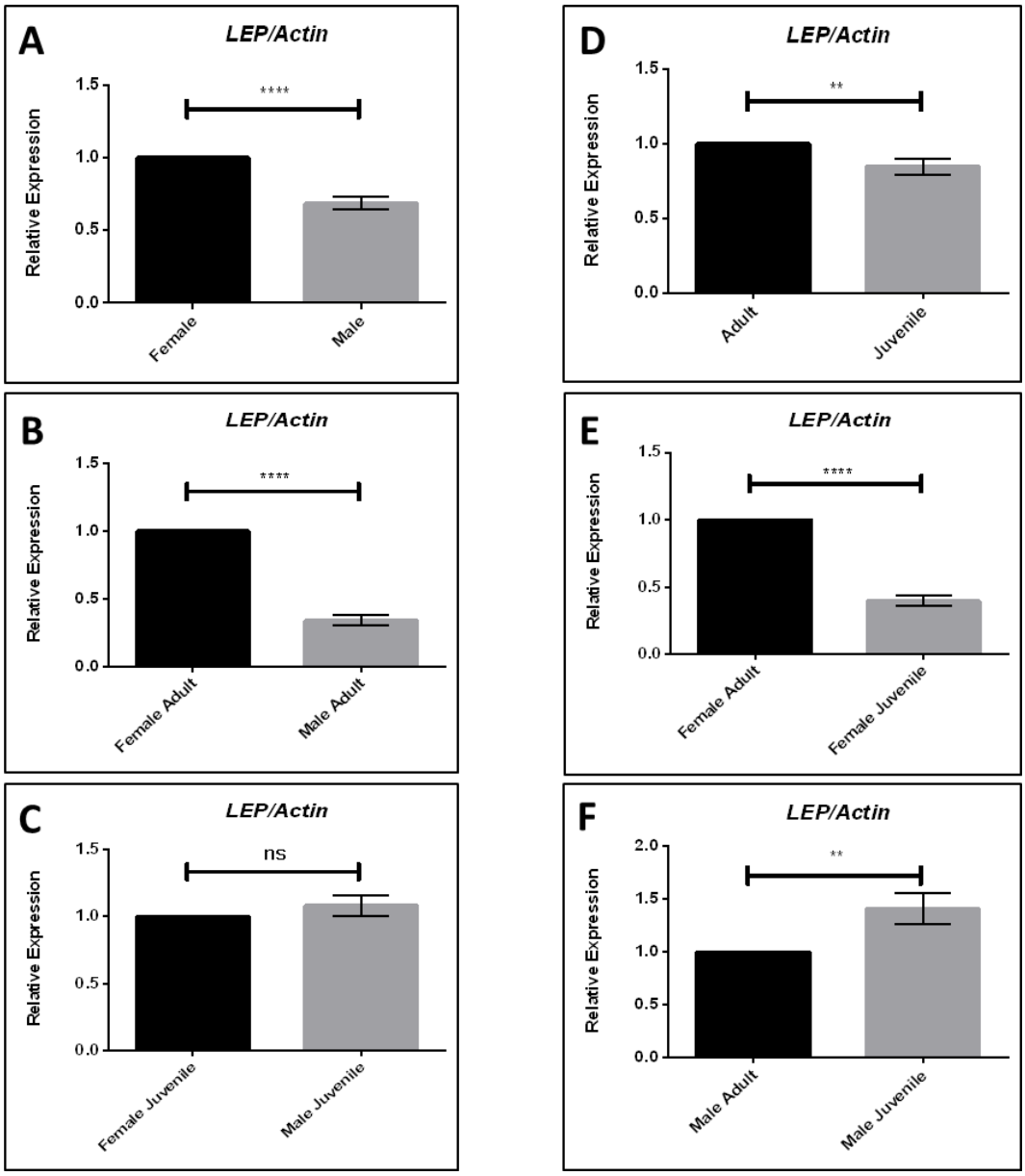


Figure 5.16. qRT-PCR validation of *LEP* between peri-renal AT of females and males, adults and juveniles of African elephants. The mRNA expression of *LEP* was higher in the females compared to the males (A), however there was an age influence as it was only statistically significant between the adults (B), but not between the juveniles (C). Comparison between age groups in both sexes showed significant differential expression, although expression was higher in the female adults (E) and lower in the male adults (F) compared to juveniles. The qRT-PCR results between adult females and males was consistent with the data obtained from RNA-seq (Table 5.9). Mean values $\pm$ SEM. Level of significance: ns = non-significant, * = $p < 0.05$, ** = $p < 0.005$, **** = $p < 0.0001$. (n=3 for each of age group and sexes).

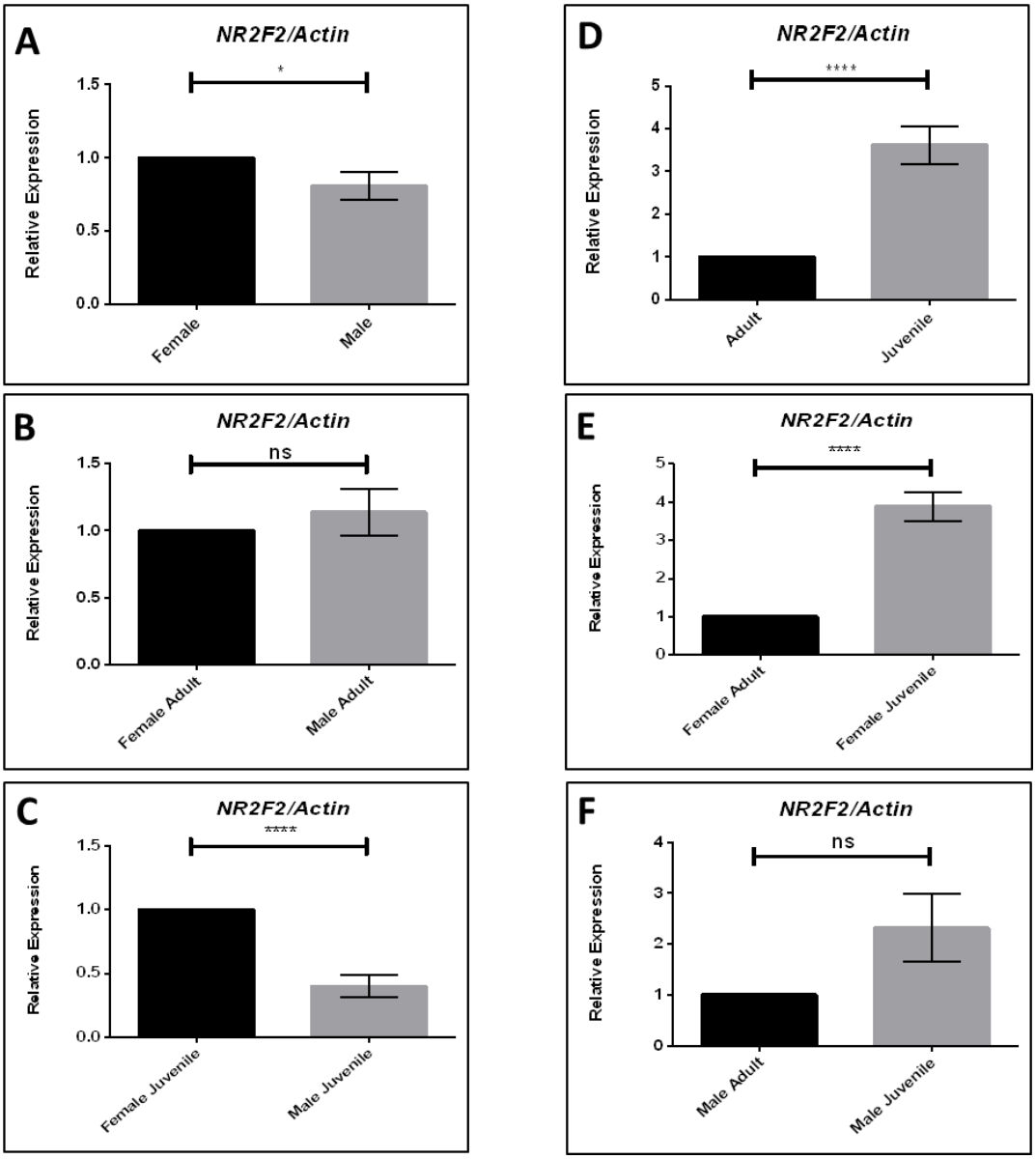


Figure 5.17. qRT-PCR validation of *NR2F2* between peri-renal AT of females and males, adults and juveniles of African elephants. The mRNA expression of *NR2F2* was significantly different between the sexes when adult and juvenile samples were analysed together using qRT-PCR (A), as well as in the juveniles (C). The results between adult females and males are not significantly different (B) and this is consistent with the data obtained from RNA-seq (Table 5.9). Comparison between age groups showed lower expressions of *NR2F2* in the adults compared to the juveniles (D), although this was only statistically significant between the females (E), and not between the males (F). Mean values $\pm$ SEM. Level of significance: ns = non-significant, * = $p < 0.05$, **** = $p < 0.0001$. (n=3 for each of age group and sexes).

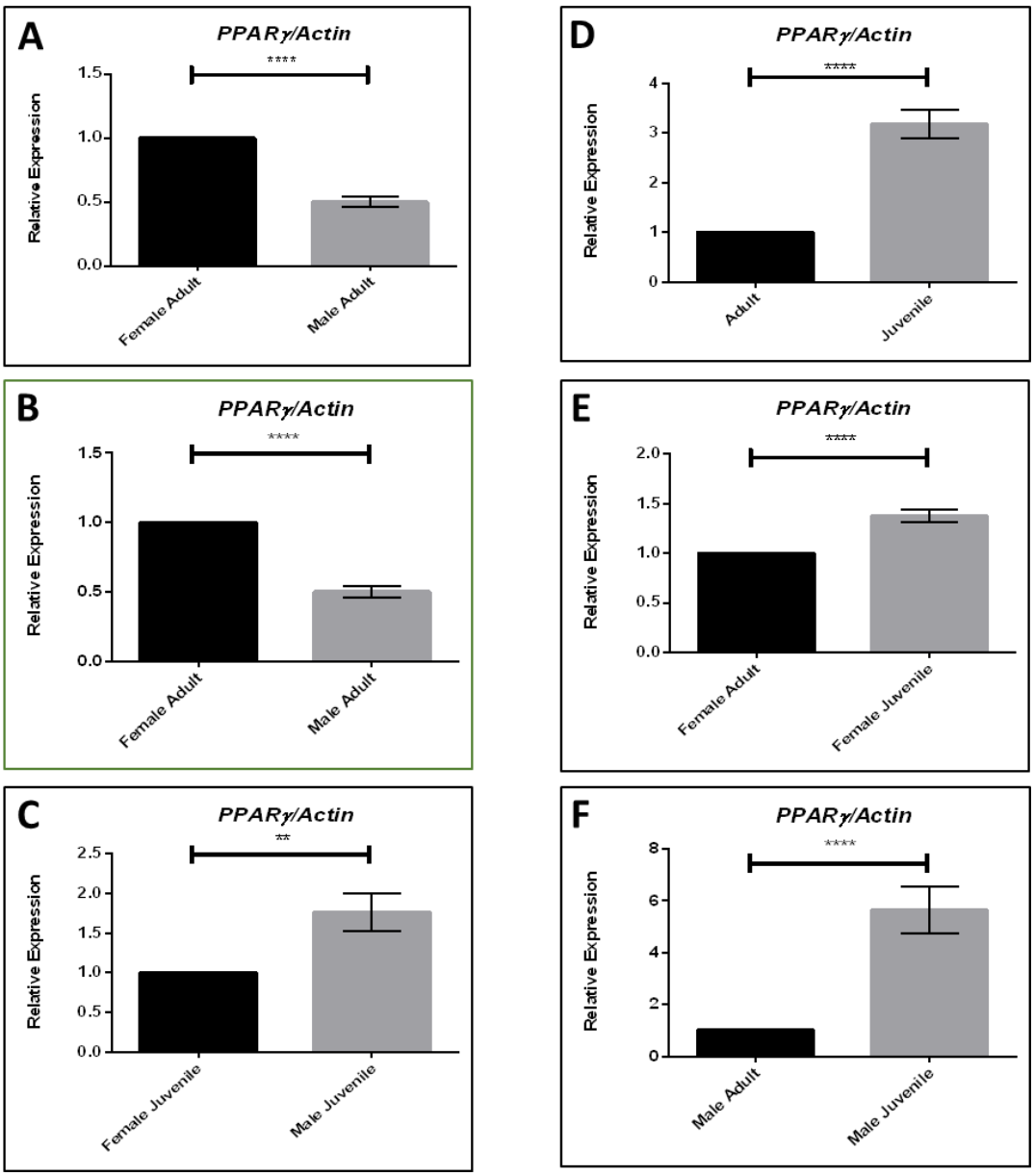


Figure 5.18. qRT-PCR validation of *PPARγ* between peri-renal AT of females and males, adults and juveniles of African elephants. The *PPARγ* mRNA expression was significantly higher in the females and males when adult and juveniles samples were analysed together (A), also between the adults (B). However, *PPARγ* mRNA expression was lower in the female juveniles compared to the males (C). Comparison between the age groups showed lower mRNA expression in the adults compared to the juveniles (D, E, and F). The qRT-PR results between adult females and males was not consistent with the data obtained from RNA-seq (Table 5.9). Mean values ± SEM. Level of significance: ** = $p < 0.005$, **** = $p < 0.0001$. (n=3 for each of age group and sexes).

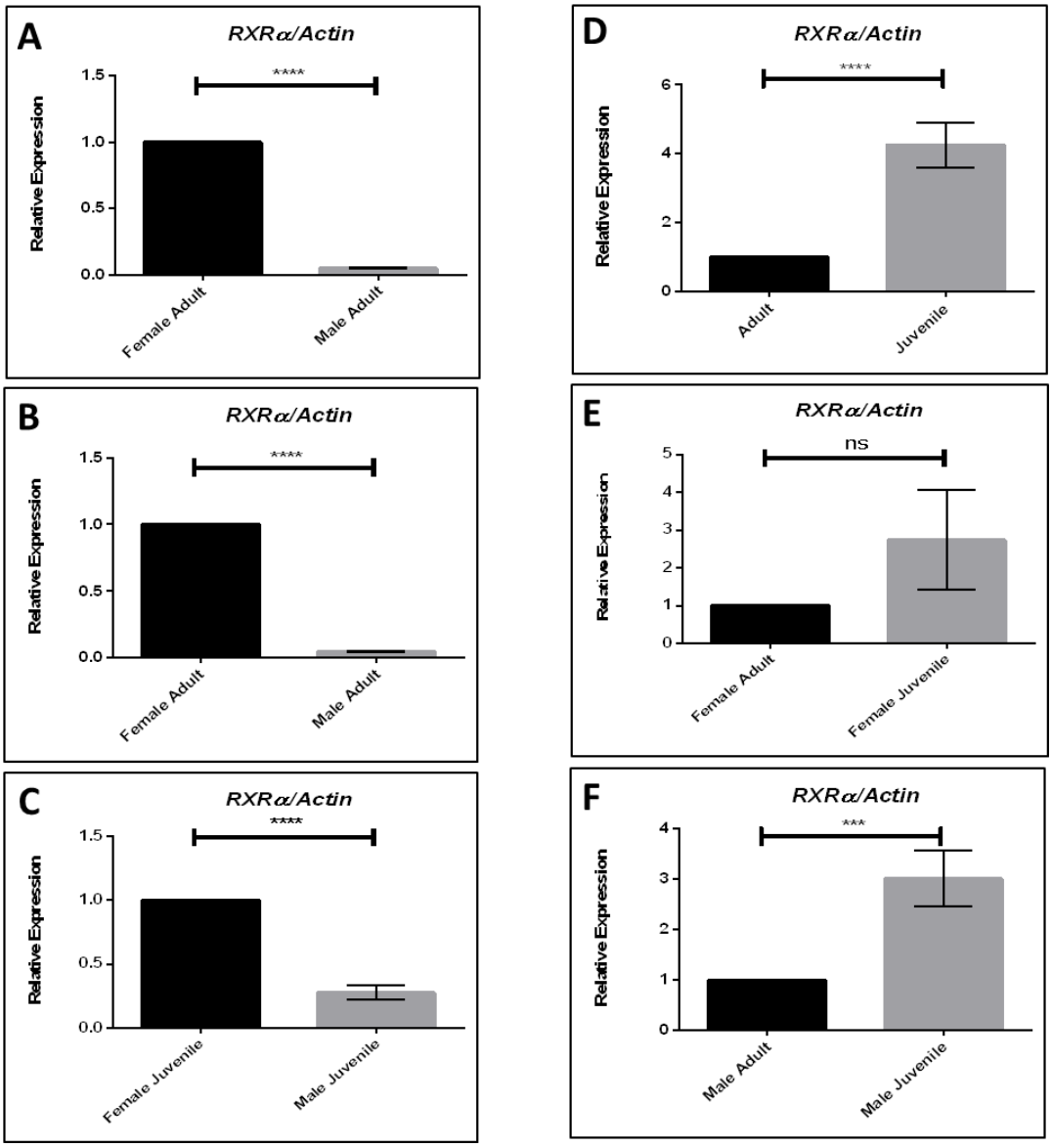


Figure 5.19. qRT-PCR validation of *RXRα* between peri-renal AT of females and males, adults and juveniles of African elephants. The mRNA expression of *RXRα* was consistently higher in the females compared to males (A, B, and C), and all adult samples exhibited lower mRNA expression than the juveniles (D, E and F). The qRT-PCR results between adult females and males was consistent with the data obtained from RNA-seq (Table 5.9). Mean values ± SEM. Level of significance: ns = non-significant, *** = $p < 0.0005$, **** = $p < 0.0001$. (n=3 for each of age group and sexes).

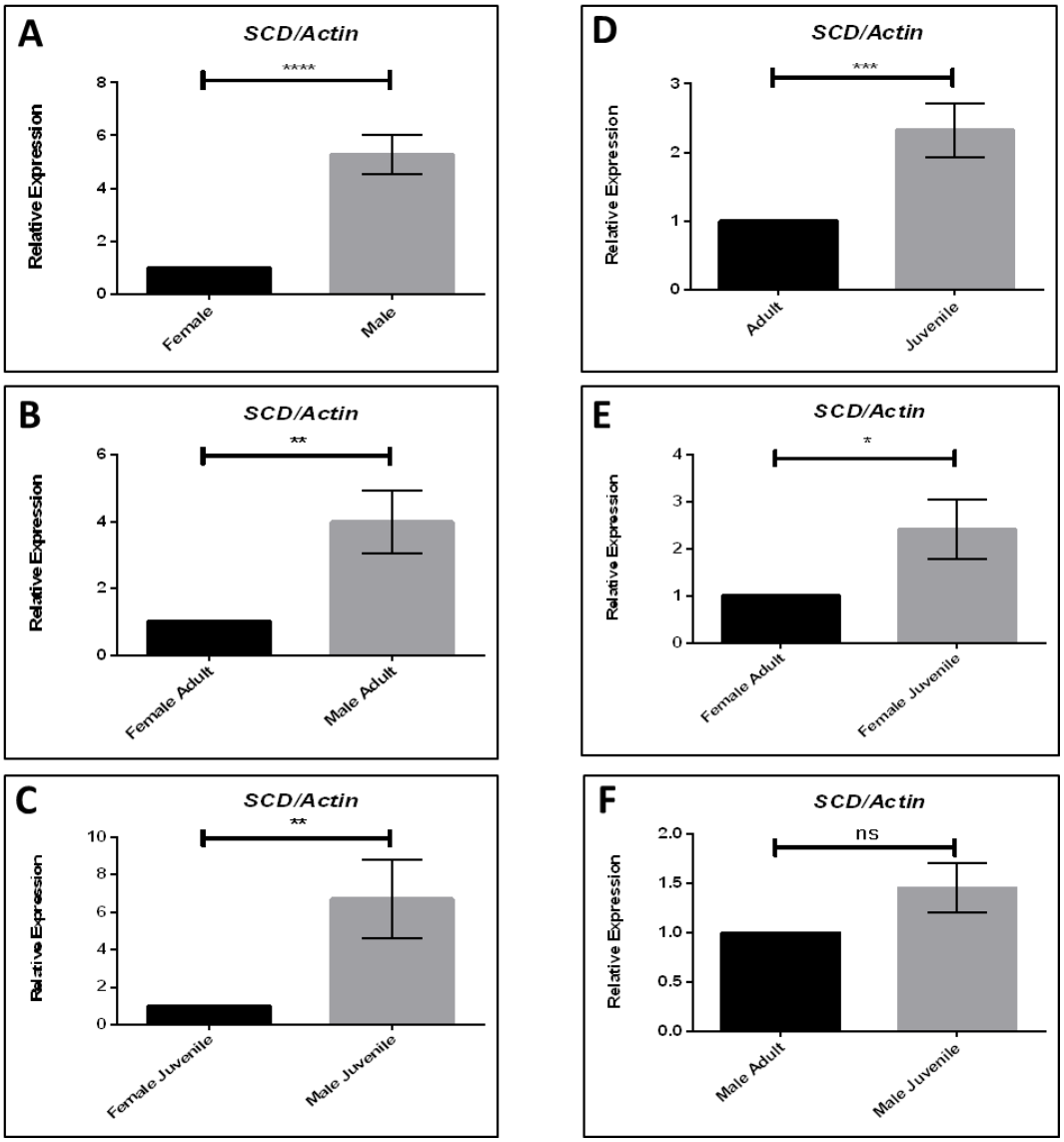


Figure 5.20. qRT-PCR validation of *SCD* between peri-renal AT of females and males, adults and juveniles of African elephants. In general, the mRNA expression of *SCD* was lower in the females compared to the males (A, B, and C), and in the adults compared to the juveniles (D, and E). Although the difference was not statistically significant between male adults and the juveniles (F). The results between adult females and males was not consistent with the data obtained from RNA-seq (Table 5.9). Mean values ± SEM. Level of significance: ns = non-significant, * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$, **** = $p < 0.0001$. (n=3 for each of age group and sexes).

5.4 Discussion

5.4.1 Animals and samples

5.4.1.2 Comparison between sexes in the African elephants

From among the seven genes of interest between AT of adult female and male elephants, four of the RNA-seq results were validated by qRT-PCR (*ELOVL5*, *LEP*, *NR2F2*, *RXRα*). Whereas the other three showed disparities between RNA-seq and qRT-PCR (*FASN*, *PPARγ*, *SCD*). This is most likely due to the differences of expression count values between the two females, where one was higher, while the other was lower than the male in RNA-seq (Table 5.9). However, results from replicates in qRT-PCR generally reached consensus within the sexes and age groups respectively. High-throughput platforms such as microarrays and RNA-seq are important tools to interrogate large quantities of data. However qRT-PCR is considered the gold standard technique for gene expression quantification due to its proven accuracy and specificity, and is often used to validate the results from microarray and RNA-seq (Wang et al., 2006, Ballester et al., 2013, Prokopec et al., 2013). Furthermore, higher number of replicates were used for qRT-PCR compared to RNA-seq in this study, which improves the statistical power of the results (Bustin et al., 2009). Thus validation of RNA-seq results by qRT-PCR is always necessary to ensure the significance and accuracy of results obtained from RNA-seq.

From morphological observations obtained during sample collection, both female samples sent for NGS were not pregnant, but Female1 was lactating. These samples were chosen as both were from non-pregnant animals, and

were presumed to exhibit similar transcriptomic signatures (in contrast to pregnant animals). Indeed these were the only two samples from non-pregnant adult females. However, differential expression (DE) analysis between the two females revealed a relatively high number of DEG between the two. This could be due to the differences of transcriptional signals from AT as a result of a higher energy expenditure channelled towards milk production in Female1. For qRT-PCR, RNA was extracted from a non-pregnant lactating adult female and two pregnant females, due to limitations of samples available (very few samples from adult non-pregnant females). This was unavoidable, as adult females in the wild are either pregnant or in lactational anoestrus, and prolonged non-conceptional oestrus is usually rare (Allen, 2006, Hildebrandt et al., 2006b). As qRT-PCR Ct values among adult females were similar, it may be that the metabolic changes occurring in AT of lactating and pregnant females rendered these sample more similar to each other than either one is to a non-pregnant female. Nevertheless, energy status of the non-pregnant female may be more similar to that of the adult male, than to the lactating female, this would explain the clustering of Female2 with the Male1 in hierarchical analysis (Figure 5.9). It is also possible that Female2 was not exhibiting normal reproductive activity, as it had only 1 placental scar, even although it was the oldest female sampled (Fiona Stansfield, pers comm). Placental scars form due to the considerable amount of maternal tissue which detaches from the uterine wall when the placenta is shed post-partum. Therefore, in the African elephant, the number of placental scars indicates the number of near or full term pregnancies the female previously experienced (Laws, 1967). As adult females are rarely in prolonged non-conceptional

oestrus (Allen, 2006, Hildebrandt et al., 2006b), Female2 should have more than one placental scar at the age of 32 years. The dominant female in a family herd (the matriarch), may experience suppressed ovarian activity in the wild (Freeman et al., 2013). However this may not be an appropriate explanation for Female2, as matriarchs are usually around 50 to 60 years old and are undergoing reproductive senescence (Freeman et al., 2013).

In the wild population, females attain sexual maturity between 8 and 12 years of age, while males tend to reach puberty 1 - 2 years after the females (Brown, 2014). Males will leave their natal herd after attaining puberty, and form bachelor herd with other males or live solitarily, a natural behaviour believed to prevent inbreeding within the herd (Douglas-Hamilton et al., 2001), However, because only family groups were culled, there were very few samples collected from adult males in this study. Brown (2014) reported successful mating by captive males as early as 6 years of age. However field observations recorded on wild elephants indicate that average male African elephants attain puberty around 10 to 12 years old, with their first mating commonly occurring at 29 years of age, and peak reproductive period occurs between 45 and 53 years old (Hollister-Smith et al., 2007). The oldest males sampled in the current study were between 8 to 16 years old. The samples from 8-year old males may not necessarily have been accurately categorised as adult samples. However, they were the only samples available which could be possibly be considered as coming from adults, as other samples were from even younger animals.

5.4.2 Transcriptome comparison in the elephants

Differential mRNA expression in peri-renal AT of male and female elephants was analysed by comparing transcription signatures between the sexes from RNA-seq data, and results were validated using qRT-PCR. Additionally, qRT-PCR was performed to compare mRNA expression between different age groups for both sexes.

5.4.2.1 Transcriptome comparison between sexes

Comparison of transcriptomes between females and males showed significantly higher mRNA expression of *FASN*, and *SCD* in adult males compared to adult females (Figures 5.15 and 5.20). On the other hand, adult females showed higher transcriptomic expression of *LEP*, *PPARγ* and *RXRα* compared to adult males (Figures 5.16, 5.18, and 5.19). However, expression levels of *ELOVL5*, and *NR2F2* were not significantly different between the adults (Figures 5.14 and 5.17). Meanwhile, juvenile females exhibited higher mRNA expression of *NR2F2* and *RXRα* (Figures 5.17 and 5.19), but lower *ELOVL5*, *FASN*, *PPARγ*, and *SCD* compared to juvenile males (Figures 5.14, 5.15, 5.18 and 5.20), and *LEP* expression was not significantly different between juveniles of different sexes (Figures 5.16).

FASN and *SCD* have been discussed in previous chapters as lipogenic factors involved in *de novo* fatty acid (FA) biosynthesis, and their expressions were elevated in AT when higher FA content was recorded (Madeira et al., 2013). Thus, this would suggest higher adiposity in males compared to the females

in elephants, which is contradictory to findings of higher adipose deposition in females than males, in humans, pigs, and cattle (Lemieux et al., 1993, Nagy et al., 1996, Gaden, 2003, Phillips, 2010, Danfær and Strathe, 2012). However, the site of fat deposition differs between sexes in humans, as men tend to store fat predominantly in the visceral depots, whereas a higher proportion of fat accumulation occurs in the subcutaneous depots in women (Lemieux et al., 1993, Nguyen et al., 1996). An investigation of visceral fat turnover in lactating Holstein cows revealed that visceral depots were mobilised to meet the high energy demand required during lactation (Pfuhl et al., 2007). Pregnant women and females rats have been reported to accumulate fat in all depots during early pregnancy, however deposition was predominantly in the abdominal visceral regions (Steingrimsdottir et al., 1980, Ramos et al., 2003, Kinoshita and Itoh, 2006). Although, in later stages of gestation, these fat depots were mobilised to meet the rapid growth of the foetus as well as to maintain maternal body condition (Ewan, 1991, Homko et al., 1999, Ramos et al., 2003, Lain and Catalano, 2007). Therefore, lower mRNA expression of *FASN* and *SCD* in the AT of adult females may be attributable to the mobilisation of their fat depots to meet the higher energy demands incurred due to lactation or pregnancy. Comparing the mRNA expression count value between Female1 (non-pregnant, lactating) and Female 2 (non-pregnant and non-lactating) further confirms this as *FASN* and *SCD* mRNA expression in Female2 was considerably higher compared to Female1 and Male1 (Table 5.9). While the lower *FASN* and *SCD* mRNA expression in female juveniles compared with the males may indicate higher lipid deposition in adipocytes of the males.

The gene *PPARγ* has been demonstrated to be crucial for adipogenesis, and in maintaining the differentiated state of adipocytes (Rosen and MacDougald, 2006). Furthermore, *PPARγ* works in concert with *RXRα* to forms a heterodimeric complex that regulate adipogenesis and lipogenesis in AT (Tontonoz et al., 1994, Tontonoz and Spiegelman, 2008). The higher expression of this gene in females may indicate that female adult elephants were actively undergoing adipogenesis in comparison to the males. Elevation of CCAAT/Enhancer Binding Protein (*C/EBPα*) in RNA-seq of the females supports this (Male versus Females, log FC = -1.45, FDR = 0.43), as *C/EBPα* is an important component promoting adipocyte differentiation by activating transcription of *PPARγ* (Rosen et al., 2002). This may be due to an increase of visceral adipose mass accretion during gestation, as reported in woman, rats and dairy cows (Steingrimsdottir et al., 1980, Kinoshita and Itoh, 2006, Einstein et al., 2008, Stuebe and Rich-Edwards, 2009). Although it appear to contradict the lower mRNA expression of *FASN* and *SCD* in adult female elephants discussed in previous paragraph, which could be a result of enhanced energy expenditure during gestation. Thus, there may be other factors that influence the expression of these genes in the African elephants. The mRNA expression of *PPARγ* was higher in female juveniles but *RXRα* was higher in the males, this may indicate that adipogenesis between the juveniles was not significantly different.

In addition to its role in energy homeostasis through regulation of food intake, *LEP* expression also relays signals to the central nervous system on the reproductive readiness of individuals based on the sufficiency of their energy reserves (Campfield et al., 1995, Campfield et al., 1996, Gao and Horvath,

2008). *LEP* was also reported to directly inhibit lipogenesis by supressing acetyl-CoA carboxylase (*ACACA*) and *FASN* expression, while stimulating FA oxidation through increased expression of acyl CoA oxidase (*ACO*) and carnitine palmitoyl transferase (CPT); (Bai et al., 1996, Wang et al., 1999a). This may partly explain the higher mRNA expression of *LEP* but lower *FASN* in adult females compared to the males, as *LEP* may be inhibiting expression of *FASN* and lipid was being channelled towards energy demand in other tissues instead of AT. In addition, *in vivo* and *in vitro* studies reported stimulatory effects of 17β-estradiol (E_2) and inhibitory effects of dihydrotestosterone (DHT) on *Lep* mRNA expression in peri-renal adipose in rats (Machinal et al., 1999). Thus E_2 in females may promote *LEP* expression, while DHT in males could inhibit it. In further support of this, it was determined that circulating LEP levels in men were consistently lower than those in women who were at equivalent levels of adiposity (Kennedy et al., 1997). Circulating plasma concentrations of LEP rise progressively in pregnant women, in parallel with an increase in their fat mass (Sattar et al., 1998). *LEP* mRNA expression was also found to be enhanced by *C/EBPα* (Krempler et al., 2000), and in the current study, elevated *C/EBPα* mRNA expression was detected in the RNA-seq results from adult females compared to the male (log FC = -1.45, FDR = 0.43, Female1 = 507, Female2 = 786, Male1 = 150). Higher *LEP* expression in adult female African elephants compared to the adult males observed in this study and in earlier publication (Nilsson et al., 2014) are consistent with results from previous studies in rats and humans. As juveniles were not sexually mature (and therefore sex hormones were at basal levels),

it is unsurprising that mRNA expression of *LEP* in juveniles did not differ between the sexes.

NR2F2 and *ELOVL5* have been reported to promote *de novo* lipogenesis and adiposity (Li et al., 2009, Viguerie et al., 2012). Therefore, DE of these genes seem likely to depend on the differing levels of adiposity and lipogenic activity in the adipose depot. The mRNA expression of these genes was not significantly different between adults of both sexes in either the RNA-seq or the qRT-PCR. This was in contrast to previous microarray results from peri-renal AT, which demonstrated higher expression of *NR2F2* in female adult African elephants compared to the adult males, which may be due to the sex-related differences in adipose deposition in elephants (Nilsson et al., 2014). However, as enhanced lipolytic activities was indicated in the female adults compared to the males, this may have reduced *NR2F2* mRNA expression in the females. Both *NR2F2* and *ELOVL5* were differentially expressed in the juveniles, although *NR2F2* was higher in females, while *ELOVL5* was higher in the males. Again, this may indicate that lipogenesis was not significantly different between the juveniles.

Overall, female adults appeared to have a higher energy expenditure and lower level of lipogenesis compared to the males. However, the juveniles appeared to be comparable to one another in terms of energy homeostasis, as mRNA expressions for both sexes did not show clear pattern of DE. This may be because the male and female juveniles were growing at a comparable pace to each other at the ages at which they were sampled. Previous studies have found that male calves will experience faster growth rate than females

from the age of four (Eltringham, 1982), while others observed evidence of differences in growth rate of males and females only after 10 years of age (Lindeque and Jaarsveld, 1993). Therefore, as the calves in this study were younger than four years of age, it may be that the difference in growth rate had not yet manifested between the two sexes.

5.4.2.2 Transcriptome comparison between adults and juveniles

A comparison of expression between adult and juvenile elephants revealed that female adults had higher mRNA expression of *ELOVL5*, *FASN*, *LEP* compared to juveniles (Figures 5.14, 5.15 and 5.16), but lower expression in *NR2F2*, *PPARγ*, and *SCD* (Figures 5.17, 5.18, and 5.20), while *RXRα* (Figure 5.19) expression was not significantly different between the female age groups. In the males, increased expression of *ELOVL5* and *FASN* was observed in the adults (Figures 5.14 and 5.15), but lower expression of *LEP*, *PPARγ*, and *RXRα* compared to the juveniles (Figures 5.16, 5.18, and 5.19). Although *NR2F2* and *SCD* expression was not significantly different between the two age groups, the juveniles were trending towards higher expression levels compared to the adults (Figures 5.17 and 5.20).

In livestock, development of bone and muscle are prioritised during the growing phase while adipose accretion occurred at the maturing or finishing stage, mainly through hypertrophy of adipocytes (Eggert et al., 1996, Mitchell et al., 2001, Gaden, 2003). Fat mass in human and rodents was also reported to expand with the advance of age (Wolden-Hanson, 2010, Bazzocchi et al., 2013). This appears to be true in African elephants as AT of both male and

female adults exhibited enhanced lipogenic activity through higher mRNA expression of *ELOVL5* and *FASN*, while the juveniles were more inclined toward adipogenesis with elevated *PPARγ* and *SCD* mRNA expressions.

It has been noted that as male mice matured, expression of *ELOVL5* increased in their muscle and this may be related to an increase in testosterone levels after reaching puberty, although the mechanism has not been elucidated (Park et al., 2013). If this also occurs in elephants, and if this difference is also present in their AT, this may explain the higher mRNA levels of *ELOVL5* in adult males compared to the juveniles. However, androgenic hormones may not be the only influence on expression of this gene, as adult females also exhibited greater *ELOVL5* mRNA expression than the juveniles. On the other hand, heightened *SCD* mRNA expression in juveniles may indicate greater Δ9-desaturase enzymatic activities, and consequently higher desaturation of SFAs, palmitic (16:0) and stearic acid (18:0), to produce MUFAs palmitoleic (16:1) and oleic acid (18:1); (Ntambi et al., 1988, Miyazaki and Ntambi, 2003). Previous work did not reveal significant differences between levels of these fatty acids (FAs) between adult and juvenile African elephants (Nilsson et al., 2014). However, this could be further examined in future experiments.

As mentioned before, *LEP* expression has been shown to be higher in women and female rats compared to males due to the stimulatory effects of estrogen, but androgenic hormones appear to exert inhibitory action on *LEP* expression (Machinal et al., 1999, Kennedy et al., 1997). In the juveniles, *LEP* mRNA expression was lower in the females, but higher in the males, compared to the adults. This is consistent with the fact that juveniles would not been secreting

the higher levels of sex steroids as produced by the adults. As a result, the juvenile females would not have experienced the stimulatory effects from estrogen, nor the juvenile males the inhibitory effects from testosterone. Studies in rats illustrated an age-related inverse relationship between *Lep* and *Fasn* mRNA expression, where *Lep* mRNA expression increased with age while *Fasn* decreased (Nogalska and Swierczynski, 2001, Nogalska and Swierczynski, 2004, Swierczynski, 2006, Wronska et al., 2014). A similar relationship was seen in expression of these genes in the adult and juvenile male elephants in the current study. However, this was not the case for the females, as adults had higher mRNA expression for both *LEP* and *FASN* compared to the juveniles. Therefore, the regulatory action of *LEP* on *FASN* in female African elephants may be more complex, involving modification by other factors.

Studies of ob/ob mice (which lack the ability to produce LEP) found that *Scd* mRNA levels were significantly elevated in the liver, but *Scd* mRNA expression was normalised to the level of control animals with LEP treatment. The experiment further demonstrated *Scd* enzymatic activity was elevated in the livers of untreated ob/ob mice compared to the wild-type, as the concentration of MUFAs (palmitoleic and oleic acids), the end products of *Scd* activity, were increased (Cohen and Friedman, 2004). If the inhibitory effect of LEP on *Scd* mRNA in rats can be extrapolated to African elephants, the lower *SCD* mRNA expression in female adult elephants relative to the juveniles could be a result of the influence of *LEP*, which may have led to the lower *SCD* mRNA expression in the adult females. However this was not observed in the males,

as higher *LEP* mRNA expression in the juveniles compared to the adults did not cause a significant DE of *SCD* gene between the age groups.

As was previously discussed in Chapter 1 Section 1.1.3*, PPARγ* and *RXRα* are key factors in promoting the differentiation and maturation of adipocytes (Tontonoz et al., 1994, Tontonoz and Spiegelman, 2008). Therefore, increased mRNA expression levels of these genes in the juveniles of both sexes suggests they are undergoing a higher degree of adipogenesis compared to the adults, most likely corresponding to the higher growth rate in the juveniles compared to adults.

In summary, an assessment of mRNA expression of the genes of interest suggests that hyperplasia may be the predominant route by which expansion of AT occurs in juvenile elephants, while lipid accumulation of adipocytes may be the predominant method for adding to AT in the adults. In other words, the adult African elephants may have had a higher adipocyte generation rate associated with hypertrophy, whereas high generation rates in the juveniles may be linked with adipocyte hyperplasia, as been reported in human (Arner et al., 2010).

5.5 Conclusion

In the current study, utilisation of RNA-seq is a novel attempt to interrogate the transcriptome signatures of AT in wild African elephants in relation to lipid metabolism, energy storage and expenditure, as well as the link between adiposity and reproductive functions. Results indicated that physiological

states of the African elephants, such as age, pregnancy, lactation, have an influence on the expression of genes involved in adipogenesis and lipogenesis. Transcriptomic comparison between sexes of the adult elephants showed higher lipogenic activities in the males compared to the females. However, previous research in human and domestic animals observed enhanced lipogenesis in females compared to males (Lemieux et al., 1993, Nagy et al., 1996, Gaden, 2003, Phillips, 2010, Danfær and Strathe, 2012). This is most likely due to the higher energy expenditure in the females as majority of the adult female samples were acquired from pregnant or lactating animals. Therefore, lipolytic activities were increased in AT of these female adults in order to meet the high energy demand of pregnancy and lactation, as was observed in human and domestic species (Ewan, 1991, Homko et al., 1999, Ramos et al., 2003, Lain and Catalano, 2007). It was not possible to compare AT samples from non-pregnant and non-lactating adult females with adult males, as adult females in the wild are rarely in prolonged period of non-conceptional oestrus (Allen, 2006, Hildebrandt et al., 2006b). Collectively, comparison of AT mRNA expressions of genes of interest between sexes in the juveniles was not significantly different. This is most likely because of their similar growth rate between the age of one to 3.5 years (Eltringham, 1982, Lindeque and Jaarsveld, 1993).

For future work, AT samples from non-pregnant or non-lactation females and sexually active males should be incorporated in order to provide a more comprehensive assessment of AT transcriptome in the African elephants. Although the results from the current work was unable to provide a positive link between adiposity and fertility in the African elephants, data obtained can

be used as baseline information to improve captive population management of these animals in different life stages. Additional comparison of AT mRNA expression between African elephants from the wild and captive population will further the understanding of energy requirement of these animals in various physiological stages, and feed formulation for the captive population can be modified to cater for their needs accordingly. This may help to solve the fertility issues faced by African elephants in captivity. However, acquisition of additional samples may not be feasible, especially from the wild elephant population. In addition, validation of mRNA expression with protein expression of the genes of interest through immunohistochemistry, ELISA or Western blotting is warranted for future work in AT transcriptomic studies of African elephants. However, the lack of appropriate and verified antibody reagents may be a challenge.

Chapter 6 Cross Species Comparison and General Conclusion

6.1 Cross species comparison of adipose tissue transcriptome

Every animal species is unique, and each is very likely to have subtle differences in their biological functions. This is clearly illustrated in Figure 6.2, showing that gene expressions from all replicates are well clustered within their respective species. Therefore, under ideal circumstances, experiments should be carried out on the particular species of interest in order to obtain more accurate results. However, alternative animal models are often used in place of the particular species of interest. A comparison of orthologous gene expression in adipose tissue (AT) from cattle, pigs and the African elephant was therefore conducted to determine the degree of gene conservation across these species.

The total number of transcripts mapped to reference genomes of each species was 24,616 in cattle, 25,322 in pigs, and 23,245 in African elephants. Ensembl identifiers for all species were converted to “one to one” orthologous human Ensembl identifiers to facilitate comparison. In the process, 31.83%, 45.53% and 33.48% of transcripts annotated to the respective species reference genomes were lost for cattle, pigs and elephants. This means that a total of 16,780 transcripts in cattle were converted to human orthologues, 13,791 in pigs, and 15,463 in African elephants. Therefore, 11,375 transcripts common in all 3 species represented 67.79%, 82.48% and 73.56% of the orthologous transcripts in each species correspondingly (Figure 6.1). It appeared that less than 50% of genes were conserved across all 3 species, although there were more common orthologous genes between African elephants and cattle

samples, compared with pigs. Correspondingly pig samples shared a higher number of orthologous genes with cattle, than with the African elephants.

The hierarchical clustering presented in Figure 6.2 gives a global view of genes expressed in AT of cattle, pigs and African elephants. Both beef and dairy cattle breeds were included in the analysis: Aberdeen Angus crosses, Charolais, Hereford, Highland and Limousin (beef), and Holstein and Jersey (dairy). The pig breeds included were Large White (LW) and Meishan (MS). The Ensembl identifiers of all genes mapped to reference genome from each species were converted to their orthologous human gene Ensembl identifiers to facilitate interspecies comparison. From a total of 16,780 orthologous genes identified through RNA-seq in cattle, 13,791 in pig, and 15,463 in the African elephant respectively, the number of genes from all species was filtered down to 2,351 with all 21 samples with read counts $\geq$ 50, and fold change (FC) ≥ 2.0. The replicates appeared to group together in their corresponding species. It was of interest to observe that Charolais (a beef breed) was clustered with the Jersey (a dairy breed). This may indicate that mRNA expression in AT of cattle may not be solely determined by the cow's main productive function of producing milk or meat. Also, this figure differs from the one in the cattle chapter (Appendix 6) most likely due to the relative substantial number of genes discarded as a result of orthologous matches. However, as AT from Charolais, and Jersey were single samples, further analysis was not conducted.

In order to test the accuracy of the hierarchical clustering, the analysis was repeated with bootstrap resampling, and the Approximately Unbiased (AU) p-

value percentages (in red) and Bootstrap Probability (BP) value percentages (in green) were calculated for each node of the dendrogram (Figure 6.3). These values showed the strength of clusters formed being supported by the database, and the higher percentages indicated a greater probability that the dendrogram was true (Shimodaira, 2002, Shimodaira, 2004). AU p-values were calculated through multiscale bootstrap resampling, while BP values were computed using normal bootstrap resampling. All replicates were confidently segregated into their respective species as shown in Figure 6.3. In both Figures 6.2 and 6.3, there were divergence between breeds of cattle, where 3 of the Aberdeen Angus cross (AAX) replicates were distant from the rest, and the Herefords were separated from one another. In addition, similar to what was described for Figure 6.2, Charolais (beef breed) was grouped with the Jersey (dairy breeds).

From Figure 6.1, it appeared that cattle and African elephants shared a larger number of expressed genes in their peri-renal AT than either did with the pigs. This may be due to the age of the animals, or to the location of the fat depot from which the samples were taken. Firstly, both cattle and African elephant samples were from animals that had reached sexual maturity, whereas the pig samples were from 7-day old neonates. Thus, it is possible that AT of sexually mature animals have different mRNA expression from the neonates. Secondly, subcutaneous AT was sampled from the pigs, while peri-renal AT was sampled in cattle and the African elephants. Subcutaneous AT was used in the pig study as insufficient visceral AT was present in the neonatal piglets at the time of sample collection (Alison Mostyn, pers comm). The AT from different depots exhibits different morphology, metabolism and functions

(Rosen and MacDougald, 2006, Gesta et al., 2007) due to the effects of transcription factors and gene expression patterns (Vidal, 2001, Vohl et al., 2004, Gesta et al., 2006, Tchkonia et al., 2007, Tchkonia et al., 2013). Therefore, this may have contributed to the gene expression differences between the pigs with cattle and the African elephants.

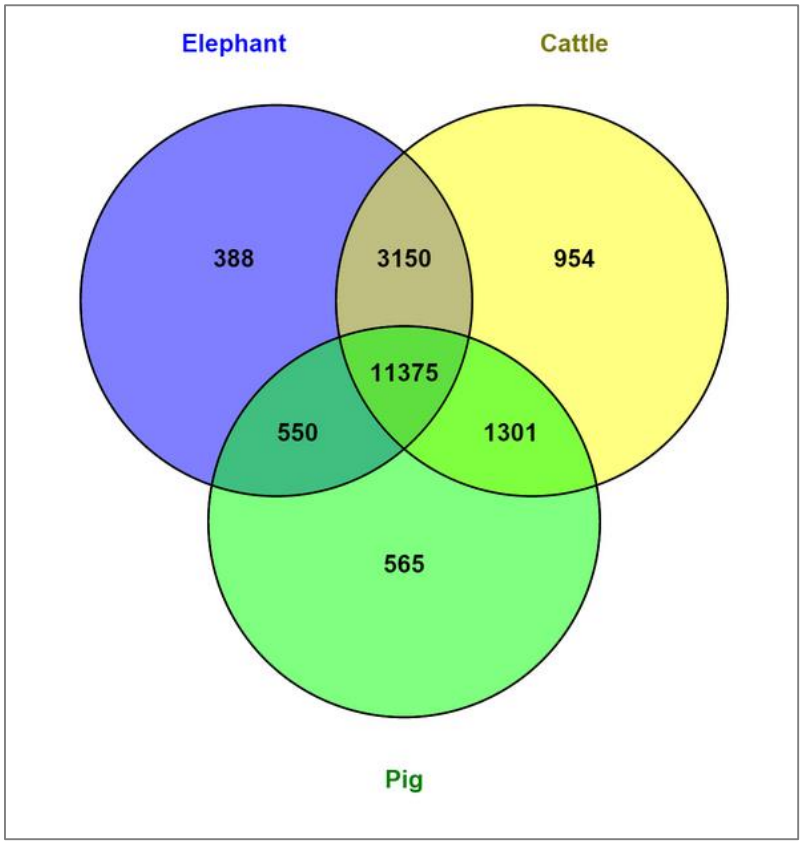


Figure 6.1. Venn diagram showing conserved genes across species expressed in AT. Ensembl identifiers for all species were converted to one-to-one orthologous human Ensembl identifiers to facilitate comparison. The 11,375 transcripts common in all 3 species represented 67.79%, 82.48% and 73.56% of the orthologous transcripts in the cattle, pigs and elephants respectively.

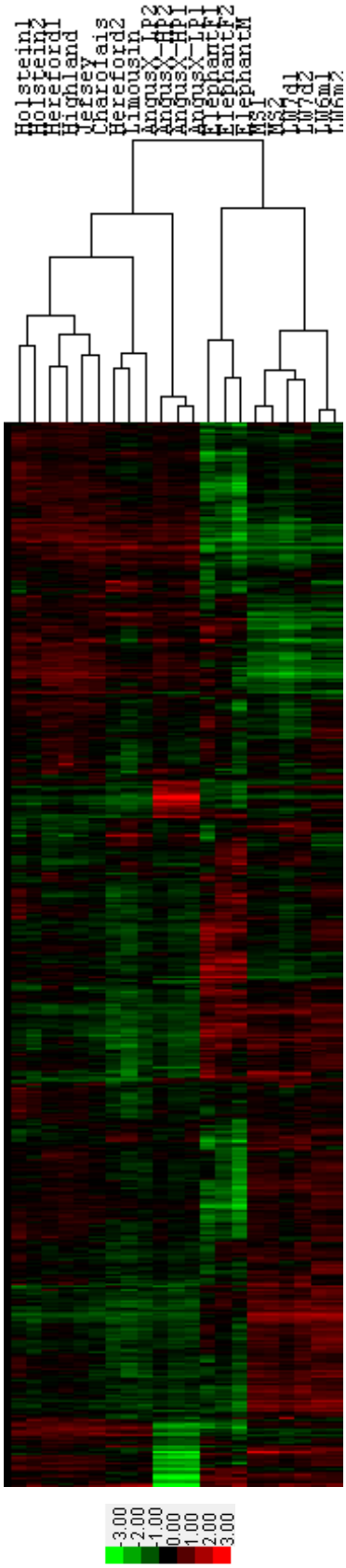


Figure 6.2. Hierarchical clustering of genes expressed in AT of cattle, pigs and African elephants. Cattle breeds included Aberdeen Angus crosses, Charolais, Hereford, Highland and Limousin (beef), and Holstein and Jersey (dairy). Pig breeds were Large White (LW) and Meishan (MS). From a total of 16,780 orthologous genes identified through RNA-seq in cattle, 13,791 in pig, and 15,463 in African elephant respectively, numbers of genes were filtered down to 2,351 with 21 samples with $\geq$ 50 read counts, and FC $\geq$ 2. (Cattle: AAX n = 4, Charolais n = 1, Hereford n = 2, Highland n=1, Holstein n = 2, Jersey n = 1, Limousin n = 1; Pigs: MS n = 2, LW n = 2; African elephants: Female n = 2, Male n = 1).

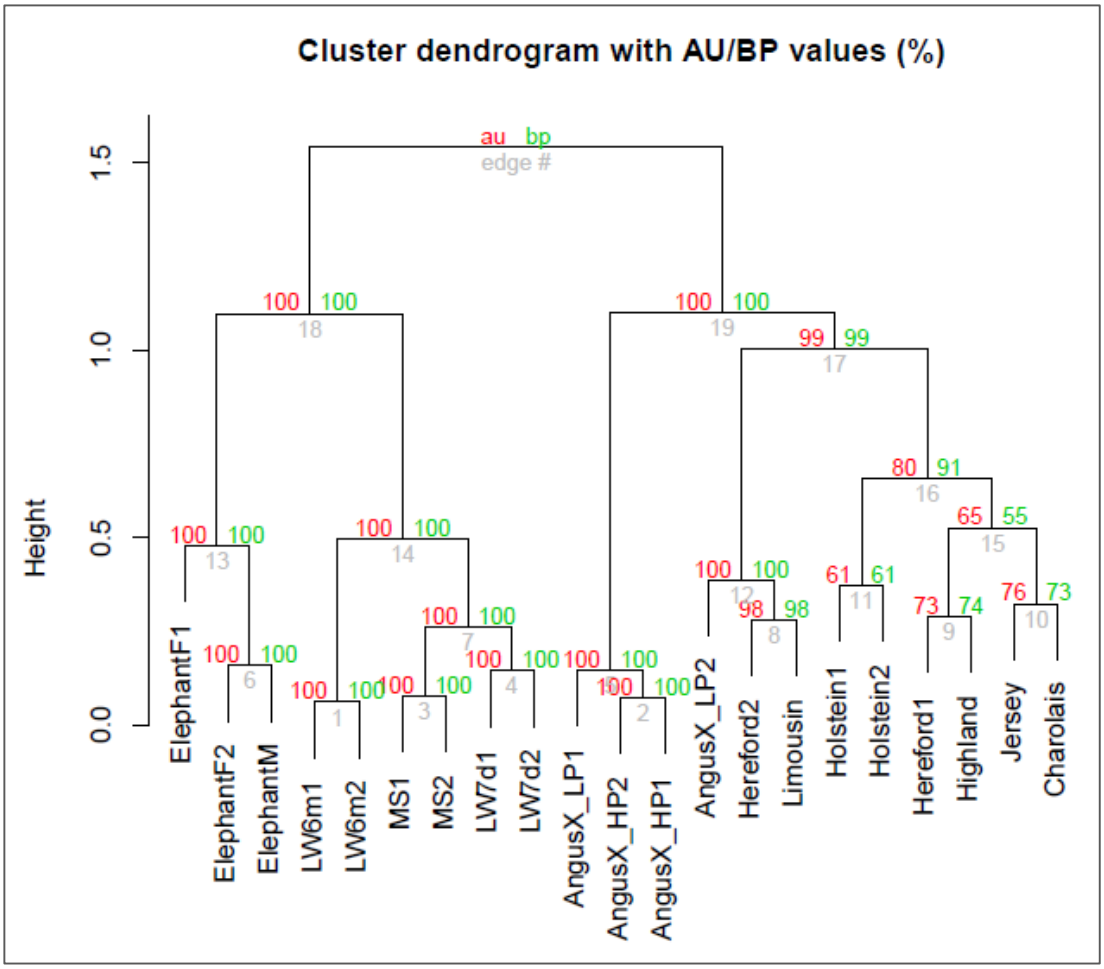


Figure 6.3. Hierarchical clustering of genes expressed in AT of cattle, pigs and African elephants with bootstrap resampling. Approximately unbiased (AU) p-value percentages (in red) and Bootstrap Probability (BP) value percentages (in green) indicated the strength of clusters formed being supported by the database.

6.2 Comparative transcriptomic analysis of adipose tissue

A comparative transcriptomic analysis of AT from different breeds in cattle and pigs in relation to adiposity, energy expenditure and reproductive performance has not been reported to date. Previous reports on transcriptome profiles of bovine and porcine AT were more descriptive of mRNA expressions rather than functional interpretation (Li et al., 2012a, Thomson et al., 2013). In the current study, whole transcriptome sequencing of AT using NGS technology in cattle, pigs and African elephants was completed to examine the AT in relation to lipid metabolism, energy storage and expenditure, also reproductive functions.

In general, RNA sequencing (RNA-seq) results from this study showed that the distinctive adiposity phenotype observed in the Hereford cattle (beef breed) compared to Holsteins (dairy breed), as well as in the MS pigs (Asian breed) compared to LW (Western breed), was supported by transcriptomic signatures related to adipogenesis and lipogenesis. This may be indirectly translated to enhanced reproductive performance in breeds with higher adiposity (Hereford and MS), compared to breeds with lower adipose deposits (Holstein and LW). Results from the current study showed that Hereford and MS are effective models to be used for comparison with Holstein and LW respectively. Distinctive transcriptomic signatures and potential mechanistic pathways that could influence energy storage and expenditure between the breeds were demonstrated.

In the adult elephants, transcriptomic comparison between the sexes revealed higher lipogenic activities in the males compared to the females. This

contradicts previous reports that observed a higher degree of lipogenesis in females compared to males in humans and domestic animals (Lemieux et al., 1993, Nagy et al., 1996, Gaden, 2003, Phillips, 2010, Danfær and Strathe, 2012). However, one possible explanation was that the majority of female samples were acquired from animals either in gestation or lactation. Therefore, AT from these females was in a lipolytic state in order to meet the high energy demand of pregnancy and lactation, as was observed in humans and domestic animal species (Ewan, 1991, Homko et al., 1999, Ramos et al., 2003, Lain and Catalano, 2007). The mRNA expressions of genes of interest from AT in the juvenile African elephants of both sexes did not appear to be significantly different from one another, this may be due to the similar growth rates at their young ages. However, AT transcriptome of 7 day old neonates in MS and LW indicated distinctive lipogenic and adipogenic activities, illustrating that the rate of AT development was already dissimilar between these breeds even at such a young age.

The leptin (*LEP*) gene or its receptor, *LEPR*, was found to be differentially expressed when compared between cattle breeds, different dietary protein levels in cattle, and between sexes of adult elephants. In contrast, *LEP* mRNA expression in AT did not vary between pig breeds and different sexes of juvenile elephants. As LEP has been associated with reproductive functions in human and animals (Clarke and Henry, 1999, Garcia et al., 2002, Barb and Kraeling, 2004, Barb et al., 2004, Barb et al., 2005, Hausman et al., 2012, Garcia-Galiano et al., 2014, Perez-Perez et al., 2015), the differential expression (DE) of both *LEP* and *LEPR* may indicate reproductive functions influence by the levels of adiposity in cattle and elephants. This was further

supported by the similar levels of *LEP* expression found in the prepubertal pigs and juvenile elephants, which had yet to attain sexual maturity.

The fatty acid (FA) composition of AT from different cattle breeds (Holstein and Hereford) and Aberdeen Angus crosses (AAX) cattle from different dietary protein levels (LP and HP) was determined using gas chromatography (GC). The percentage of saturated fatty acids (SFAs) was higher in Hereford compared to Holstein, and higher in LP compared to HP. Consequently monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) levels were lower in Hereford and LP, relative to that of Holstein and HP. As SFAs are the end products of biohydrogenation (Buccioni et al., 2012), higher SFAs content in Herefords and LP may indicate that there is a surplus of FAs and this could have enhanced adipose deposition in these breeds compared to Holsteins and HP. However, profiles of FAs through hierarchical clustering were unable to explicitly differentiate between AT of different cattle breeds, or between LP and HP. The significantly different percentages of linoleic acid (LA) and linolenic acid (LNA) between breeds and dietary protein levels may not be relevant in understanding FA metabolism in AT as these FAs had to have been from exogenous sources, as animals are unable to synthesise these essential fatty acids (Simopoulos, 1991).

By inference from research in humans and other species, outcomes from this study indicated positive and negative influence of adiposity on fertility (Frisch, 1984, Day et al., 1986, Armstrong and Britt, 1987, Frisch, 1987, Tatman et al., 1990, Wade and Schneider, 1992, Wade and Jones, 2004). Therefore productivity of cattle and pigs, as well as reproductive performance of African

elephants could be improved through research in this area. Consequently, this supports the hypothesis of the current study that the AT transcriptome will have molecular signatures that influence reproductive functions in animals.

6.3 Future research

Future work in AT transcriptome studies in the cattle, pigs and African elephants should include validation of mRNA expression with protein expression of the genes of interest using immunohistochemistry, ELISA or Western blotting. However, one challenge to this approach relates to the lack of appropriate and verified antibody reagents in these species.

In cattle, determination of the protein digestibility in the rumen should be address in future dietary protein trials, in addition to assessing different levels of protein in the diet, as protein digestibility may affect the amount of protein uptake and utilisation in cattle, thus influencing the degree of adiposity in AT. Future studies into the FA profiles of other biologically relevant tissues including milk and muscle are warranted, to ascertain if quantitative percentages of FAs in AT are similar to other tissues, thus a more complete picture of the dynamics of FA composition could be drawn, and knowledge on uptake and utilisation of AT in cattle could be further advanced.

Results from AT transcriptomic analysis in the pigs indicated that vitamin A and D could affect adiposity, and this may have an impact on reproductive performance. Therefore, further investigation into the effects of nutritional intervention on adipogenesis and lipogenesis in pigs is warranted. RNA-seq

results from the current study did not exhibit mRNA expression directly related to reproduction as the adipose samples were obtained from piglets that have yet to reach sexual maturity. Thus, comparative transcriptome analysis of sexually matured pigs would extend the knowledge obtained from results generated in the neonates.

To further the findings from the African elephants, future transcriptomic analysis of AT in the African elephants should incorporate sexually active males and non-pregnant or non-lactation females in order to provide a more comprehensive assessment of the AT transcriptome in African elephants, as this would advance the understanding of physiological processes occurring in AT of this species. However, it may not be feasible to obtain such samples, especially in the wild elephant population.

References

ABOUHEIF, E., AKAM, M., DICKINSON, W. J., HOLLAND, P. W. H., MEYER, A., PATEL, N. H., RAFF, R. A., LOUISE ROTH, V. & WRAY, G. A. 1997. Homology and developmental genes. *Trends in Genetics,* 13**,** 432-433.

ABPLANALP, J., LACZKO, E., PHILP, N. J., NEIDHARDT, J., ZUERCHER, J., BRAUN, P., SCHORDERET, D. F., MUNIER, F. L., VERREY, F., BERGER, W., CAMARGO, S. M. & KLOECKENER-GRUISSEM, B. 2013. The cataract and glucosuria associated monocarboxylate transporter MCT12 is a new creatine transporter. *Hum Mol Genet,* 22**,** 3218-26.

AFESG. 2013. *Elephant Database: Continental Totals - Provisional African Elephant Population Estimates: update to 31 Dec 2013* [Online]. Cambridge, UK: IUCN. [Accessed 15 July 2015 2015].

AFFYMETRIX. 2015. *GeneChip porcine genome array* [Online]. Affimetrix Inc. Available: http://www.affymetrix.com/catalog/131488/AFFY/Porcine-Genome-Array#1_1 [Accessed 16 January 2015 2015].

AGILENT. 2015a. *Bovine gene expression microarray* [Online]. Santa Clara, CA: Agilent. [Accessed 4 September 2015 2015].

AGILENT. 2015b. *Porcine gene expression microarray* [Online]. Agilent Technologies. Available: http://www.genomics.agilent.com/article.jsp?pageId=1508 [Accessed 16 Jan 2015 2015].

AH MEW, N., LANPHER, B. C., GROPMAN, A., CHAPMAN, K. A., SIMPSON, K. L., UREA CYCLE DISORDERS, C. & SUMMAR, M. L. 1993. Urea Cycle Disorders Overview. *In:* PAGON, R. A., ADAM, M. P., ARDINGER, H. H., WALLACE, S. E., AMEMIYA, A., BEAN, L. J. H., BIRD, T. D., DOLAN, C. R., FONG, C. T., SMITH, R. J. H. & STEPHENS, K. (eds.) *GeneReviews®.* Seattle, Washington: University of Washington, Seattle.

AHDB 2008. The technical basis and development of classification. *Review of the EU carcass classification system for beef and sheep. A report for DEFRA.* Warwickshire, UK: Agriculture and Horticulture Development Board.

AHERNE, F. X. & KIRKWOOD, R. N. 1985. Nutrition and sow prolificacy. *J Reprod Fertil Suppl,* 33**,** 169-83.

AHERNE, F. X. & WILLIAMS, I. H. 1992. Nutrition for optimizing breeding herd performance. *Vet Clin North Am Food Anim Pract,* 8**,** 589-608.

AHIMA, R. S. & FLIER, J. S. 2000. Adipose tissue as an endocrine organ. *Trends Endocrinol Metab,* 11**,** 327-32.

AKERBLAD, P., LIND, U., LIBERG, D., BAMBERG, K. & SIGVARDSSON, M. 2002. Early B-cell factor (O/E-1) is a promoter of adipogenesis and involved in control of genes important for terminal adipocyte differentiation. *Mol Cell Biol,* 22**,** 8015-25.

AL-DWAIRI, A., PABONA, J. M. P., SIMMEN, R. C. M. & SIMMEN, F. A. 2012. Cytosolic Malic Enzyme 1 (ME1) Mediates High Fat Diet-Induced Adiposity, Endocrine Profile, and Gastrointestinal Tract Proliferation-Associated Biomarkers in Male Mice. *PLoS ONE,* 7**,** e46716.

ALBRECHT, E., KOMOLKA, K., KUZINSKI, J. & MAAK, S. 2012. Agouti Revisited: Transcript Quantification of the ASIP Gene in Bovine Tissues Related to Protein Expression and Localization. *PLoS ONE,* 7**,** e35282.

ALEXANDER, G., BENNETT, J. W. & GEMMELL, R. T. 1975. Brown adipose tissue in the new-born calf (Bos taurus). *The Journal of Physiology,* 244**,** 223-234.

ALLEN, W. R. 2006. Ovulation, pregnancy, placentation and husbandry in the African elephant (Loxodonta africana). *Philos Trans R Soc Lond B Biol Sci,* 361**,** 821-34.

AMSTALDEN, M., GARCIA, M. R., STANKO, R. L., NIZIELSKI, S. E., MORRISON, C. D., KEISLER, D. H. & WILLIAMS, G. L. 2002. Central infusion of recombinant ovine leptin normalizes plasma insulin and stimulates a novel hypersecretion of luteinizing hormone after short-term fasting in mature beef cows. *Biol Reprod,* 66**,** 1555-61.

ANDERS, S., PYL, P. T. & HUBER, W. 2014. HTSeq-a Python framework to work with high-throughput sequencing data. *Bioinformatics*.

ANDREWS, Z. B., ERION, D. M., BEILER, R., CHOI, C. S., SHULMAN, G. I. & HORVATH, T. L. 2010. Uncoupling protein-2 decreases the lipogenic actions of ghrelin. *Endocrinology,* 151**,** 2078-86.

ANGE, K., CRISSEY, S. D., DOYAL, C., LANCE, K. & HINTZ, H. A survey of African (*Loxodonta Africana*) and Asian (*Elephas maximus*) elephant diets and measured body dimensions compared to their estimated nutrient requirements. *In:* EDWARDS, M., LISI, K. J., SCHLEGEL, M. L. & BRAY, R. E., eds. Fourth Conference on Zoo and Wildlife Nutrition, 2001 Lake Buena Vista, FL, USA. AZA Nutrition Advisory Group.

APHA 2015. Incidence of TB in cattle in Great Britain - GB dataset. *TB in Cattle in Great Britain.* 12 August 2015 ed. Surrey, UK: Animal and Plant Health Agency.

ARASE, K., FISLER, J. S., SHARGILL, N. S., YORK, D. A. & BRAY, G. A. 1988. Intracerebroventricular infusions of 3-OHB and insulin in a rat model of dietary obesity. *Am J Physiol,* 255**,** R974-81.

ARCHER, J. A., ARTHUR, P. F., PARNELL, P. F. & VAN DE VEN, R. J. 1998. Effect of divergent selection for yearling growth rate on female reproductive performance in Angus cattle. *Livestock Production Science,* 57**,** 33-40.

ARMSTRONG, J. D. & BRITT, J. H. 1987. Nutritionally-induced anestrus in gilts: metabolic and endocrine changes associated with cessation and resumption of estrous cycles. *J Anim Sci,* 65**,** 508-23.

ARNER, E., WESTERMARK, P. O., SPALDING, K. L., BRITTON, T., RYDÉN, M., FRISÉN, J., BERNARD, S. & ARNER, P. 2010. Adipocyte Turnover: Relevance to Human Adipose Tissue Morphology. *Diabetes,* 59**,** 105-109.

ARNER, P., HELLSTROM, L., WAHRENBERG, H. & BRONNEGARD, M. 1990. Beta-adrenoceptor expression in human fat cells from different regions. *J Clin Invest,* 86**,** 1595-600.

ARUGA, J. & MIKOSHIBA, K. 2003. Identification and characterization of Slitrk, a novel neuronal transmembrane protein family controlling neurite outgrowth. *Mol Cell Neurosci,* 24**,** 117-29.

ASHWORTH, C. J., HALEY, C. S., AITKEN, R. P. & WILMUT, I. 1990. Embryo survival and conceptus growth after reciprocal embryo transfer between Chinese Meishan and Landrace x Large White gilts. *J Reprod Fertil,* 90**,** 595-603.

ASHWORTH, C. J., PICKARD, A. R., MILLER, S. J., FLINT, A. P. & DIEHL, J. R. 1997. Comparative studies of conceptus-endometrial interactions in Large White x Landrace and Meishan gilts. *Reprod Fertil Dev,* 9**,** 217-25.

ASHWORTH, C. J., ROSS, A. W. & HALEY, C. S. 1994. Comparisons between peripheral progesterone concentrations in cyclic and pregnant Landrace x large White and Meishan gilts. *Reprod Fertil Dev,* 6**,** 777-82.

ASTIZ, S., GONZALEZ-BULNES, A., PEREZ-SOLANA, M. L., SANCHEZ-SANCHEZ, R. & TORRES-ROVIRA, L. 2013. In vitro release of ovarian progesterone is decreased during the oestrous cycle and pregnancy of swine with obesity/leptin resistance. *Reprod Domest Anim,* 48**,** e44-8.

ATCHESON, E., HAMILTON, E., PATHMANATHAN, S., GREER, B., HARRIOTT, P. & TIMSON, D. J. 2011. IQ-motif selectivity in human IQGAP2 and IQGAP3: binding of calmodulin and myosin essential light chain. *Biosci Rep,* 31**,** 371-9.

ATTIG, L., DJIANE, J., GERTLER, A., RAMPIN, O., LARCHER, T., BOUKTHIR, S., ANTON, P. M., MADEC, J. Y., GOURDOU, I. & ABDENNEBI-NAJAR, L. 2008. Study of hypothalamic leptin receptor expression in low-birth-weight piglets and effects of leptin supplementation on neonatal growth and development. *Am J Physiol Endocrinol Metab,* 295**,** E1117-25.

BAGCHI, M. K., MANTENA, S. R., KANNAN, A. & BAGCHI, I. C. 2006. Control of uterine cell proliferation and differentiation by C/EBPbeta: functional implications for establishment of early pregnancy. *Cell Cycle,* 5**,** 922-5.

BAI, Y., ZHANG, S., KIM, K. S., LEE, J. K. & KIM, K. H. 1996. Obese gene expression alters the ability of 30A5 preadipocytes to respond to lipogenic hormones. *J Biol Chem,* 271**,** 13939-42.

BAKER, D. J., JEGANATHAN, K. B., CAMERON, J. D., THOMPSON, M., JUNEJA, S., KOPECKA, A., KUMAR, R., JENKINS, R. B., DE GROEN, P. C., ROCHE, P. & VAN DEURSEN, J. M. 2004. BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. *Nat Genet,* 36**,** 744-749.

BALDWIN, R. L. V., MCLEOD, K. R. & CAPUCO, A. V. 2004. Visceral tissue growth and proliferation during the bovine lactation cycle. *Journal of Dairy Science,* 87**,** 2977-2986.

BALLESTER, M., CORDÓN, R. & FOLCH, J. M. 2013. DAG Expression: High-Throughput Gene Expression Analysis of Real-Time PCR Data Using Standard Curves for Relative Quantification. *PLoS ONE,* 8**,** e80385.

BALMFORD, A., LEADER-WILLIAMS, N. & GREEN, M. J. B. 1995. Parks or arks: where to conserve threatened mammals? *Biodiversity & Conservation,* 4**,** 595-607.

BARAK, Y., NELSON, M. C., ONG, E. S., JONES, Y. Z., RUIZ-LOZANO, P., CHIEN, K. R., KODER, A. & EVANS, R. M. 1999. PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell,* 4**,** 585-95.

BARAN, I. & GANEA, C. 2013. RyR3 in situ regulation by Ca(2+) and quercetin and the RyR3-mediated Ca(2+) release flux in intact Jurkat cells. *Arch Biochem Biophys,* 540**,** 145-59.

BARB, C. R., BARRETT, J. B. & KRAELING, R. R. 2004. Role of leptin in modulating the hypothalamic-pituitary axis and luteinizing hormone secretion in the prepuberal gilt. *Domest Anim Endocrinol,* 26**,** 201-14.

BARB, C. R., HAUSMAN, G. J. & CZAJA, K. 2005. Leptin: a metabolic signal affecting central regulation of reproduction in the pig. *Domest Anim Endocrinol,* 29**,** 186-92.

BARB, C. R., HAUSMAN, G. J. & HOUSEKNECHT, K. L. 2001. Biology of leptin in the pig. *Domestic Animal Endocrinology,* 21**,** 297-317.

BARB, C. R., HAUSMAN, G. J. & LENTS, C. A. 2008. Energy metabolism and leptin: effects on neuroendocrine regulation of reproduction in the gilt and sow. *Reprod Domest Anim,* 43 Suppl 2**,** 324-30.

BARB, C. R., HAUSMAN, G. J. & REKAYA, R. 2006. Gene expression in the brain-pituitary adipose tissue axis and luteinising hormone secretion during pubertal development in the gilt. *Soc Reprod Fertil Suppl,* 62**,** 33-44.

BARB, C. R. & KRAELING, R. R. 2004. Role of leptin in the regulation of gonadotropin secretion in farm animals. *Anim Reprod Sci,* 82-83**,** 155-67.

BARJA-FERNANDEZ, S., FOLGUEIRA, C., SEOANE, L. M., CASANUEVA, F. F., DIEGUEZ, C., CASTELAO, C., AGUERA, Z., BANOS, R., BOTELLA, C., DE LA TORRE, R., FERNANDEZ-GARCIA, J. C., FERNANDEZ-REAL, J. M., FRUHBECK, G., GOMEZ-AMBROSI, J., JIMENEZ-MURCIA, S., TINAHONES, F. J., ESTIVILL, X., FERNANDEZ-ARANDA, F. & NOGUEIRAS, R. 2015. Circulating Betatrophin Levels Are Increased in Anorexia and Decreased in Morbidly Obese Women. *J Clin Endocrinol Metab,* 100**,** E1188-96.

BATTISTELLA, M., PELTRE, B. & CRIBIER, B. 2014. PHLDA1, a follicular stem cell marker, differentiates clear-cell/granular-cell trichoblastoma and clear-cell/granular cell basal cell carcinoma: a case-control study, with first description of granular-cell trichoblastoma. *Am J Dermatopathol,* 36**,** 643-50.

BAUER-KREISEL, P., GOEPFERICH, A. & BLUNK, T. 2010. Cell-delivery therapeutics for adipose tissue regeneration. *Adv Drug Deliv Rev,* 62**,** 798-813.

BAZER, F. W., THATCHER, W. W., MARTINAT-BOTTE, F. & TERQUI, M. 1988a. Conceptus development in Large White and prolific Chinese Meishan pigs. *J Reprod Fertil,* 84**,** 37-42.

BAZER, F. W., THATCHER, W. W., MARTINAT-BOTTE, F. & TERQUI, M. 1988b. Sexual maturation and morphological development of the reproductive tract in large white and prolific Chinese Meishan pigs. *J Reprod Fertil,* 83**,** 723-8.

BAZZOCCHI, A., DIANO, D., PONTI, F., ANDREONE, A., SASSI, C., ALBISINNI, U., MARCHESINI, G. & BATTISTA, G. 2013. Health and ageing: a cross-sectional study of body composition. *Clin Nutr,* 32**,** 569-78.

BENJAMINI, Y. & HOCHBERG, Y. 1995. Controlling the False Discovery Rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B (Methodology),* 57**,** 289-300.

BENJAMINI, Y. & YEKUTIELI, D. 2001. The control of the false discovery rate in multiple testing under dependency. *The Annals of Statistics,* 29**,** 1165–1188

BERG, F., GUSTAFSON, U. & ANDERSSON, L. 2006. The Uncoupling Protein 1 Gene (UCP1) Is Disrupted in the Pig Lineage: A Genetic Explanation for Poor Thermoregulation in Piglets. *PLoS Genet,* 2**,** e129.

BERRY, D. C. & NOY, N. 2009. All-trans-retinoic acid represses obesity and insulin resistance by activating both peroxisome proliferation-activated receptor beta/delta and retinoic acid receptor. *Mol Cell Biol,* 29**,** 3286-96.

BERRY, D. C., SOLTANIAN, H. & NOY, N. 2010. Repression of cellular retinoic acid-binding protein II during adipocyte differentiation. *J Biol Chem,* 285**,** 15324-32.

BERTRAND, H. A., LYND, F. T., MASORO, E. J. & YU, B. P. 1980. Changes in adipose mass and cellularity through the adult life of rats fed ad libitum or a life-prolonging restricted diet. *J Gerontol,* 35**,** 827-35.

BERTRAND, H. A., STACY, C., MASORO, E. J., YU, B. P., MURATA, I. & MAEDA, H. 1984. Plasticity of fat cell number. *J Nutr,* 114**,** 127-31.

BEYGA, K. & REKIEL, A. 2010. The effect of body condition of late pregnant sows on fat reserves at farrowing and weaning on litter performance. *ArchivTierzucht,* 53**,** 50-64

BHAT, M., NOOLU, B., QADRI, S. S. Y. H. & ISMAIL, A. 2014. Vitamin D deficiency decreases adiposity in rats and causes altered expression of uncoupling proteins and steroid receptor coactivator3. *The Journal of Steroid Biochemistry and Molecular Biology,* 144, Part B**,** 304-312.

BIDANEL, J. P., CARITEZ, J. C. & LEGAULT, C. 1990. Ten years of experiment with Chinese pigs in France. 1. Breed evaluation. *Pig News and Information,* 11**,** 345-348.

BIDDINGER, S. B., MIYAZAKI, M., BOUCHER, J., NTAMBI, J. M. & KAHN, C. R. 2006. Leptin suppresses stearoyl-CoA desaturase 1 by mechanisms independent of insulin and sterol regulatory element-binding protein-1c. *Diabetes,* 55**,** 2032-41.

BIENSEN, N. J., WILSON, M. E. & FORD, S. P. 1998. The impact of either a Meishan or Yorkshire uterus on Meishan or Yorkshire fetal and placental development to days 70, 90, and 110 of gestation. *Journal of Animal Science,* 76**,** 2169-2176.

BIENSEN, N. J., WILSON, M. E. & FORD, S. P. 1999. The impacts of uterine environment and fetal genotype on conceptus size and placental vascularity during late gestation in pigs. *J Anim Sci,* 77**,** 954-9.

BILLON, N. & DANI, C. 2012. Developmental origins of the adipocyte lineage: new insights from genetics and genomics studies. *Stem Cell Rev,* 8**,** 55-66.

BILLON, N., IANNARELLI, P., MONTEIRO, M. C., GLAVIEUX-PARDANAUD, C., RICHARDSON, W. D., KESSARIS, N., DANI, C. & DUPIN, E. 2007. The generation of adipocytes by the neural crest. *Development,* 134**,** 2283-92.

BILLON, N., MONTEIRO, M. C. & DANI, C. 2008. Developmental origin of adipocytes: new insights into a pending question. *Biol Cell,* 100**,** 563-75.

BLANC, J. 2008. *Loxodonta africana* [Online]. Cambridge, UK: International Union for Conservation of Nature (IUCN). [Accessed 15 July 2015].

BLANC, J. J., BARNES, R. F. W., CRAIG, G. C., DUBLIN, H. T., THOULESS, C. R., DOUGLAS-HAMILTON, I. & HART, J. A. 2007. *African Elephant Status Report 2007: an update from the African Elephant Database,* Gland,Switzerland, IUCN.

BLOCK, S. S., BUTLER, W. R., EHRHARDT, R. A., BELL, A. W., VAN AMBURGH, M. E. & BOISCLAIR, Y. R. 2001. Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. *J Endocrinol,* 171**,** 339-48.

BLOCK, S. S., RHOADS, R. P., BAUMAN, D. E., EHRHARDT, R. A., MCGUIRE, M. A., CROOKER, B. A., GRIINARI, J. M., MACKLE, T. R., WEBER, W. J., VAN AMBURGH, M. E. & BOISCLAIR, Y. R. 2003. Demonstration of a role for insulin in the regulation of leptin in lactating dairy cows. *J Dairy Sci,* 86**,** 3508-15.

BLONDIN, P., BOUSQUET, D., TWAGIRAMUNGU, H., BARNES, F. & SIRARD, M. A. 2002. Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biol Reprod,* 66**,** 38-43.

BLUMBERG, J. M., TZAMELI, I., ASTAPOVA, I., LAM, F. S., FLIER, J. S. & HOLLENBERG, A. N. 2006. Complex role of the vitamin D receptor and its ligand in adipogenesis in 3T3-L1 cells. *J Biol Chem,* 281**,** 11205-13.

BOLET, G., BOTTE, F. M., LOCATELLI, A., GRUAND, J., TERQUI, M. & BERTHELOT, F. 1986. Components of prolificacy in hyperprolific Large White sows compared with the Meishan and Large White breeds. *Genet Sel Evol,* 18**,** 333-42.

BOLINDER, J., KAGER, L., OSTMAN, J. & ARNER, P. 1983. Differences at the receptor and postreceptor levels between human omental and subcutaneous adipose tissue in the action of insulin on lipolysis. *Diabetes,* 32**,** 117-23.

BONET, M. L., OLIVER, J., PICO, C., FELIPE, F., RIBOT, J., CINTI, S. & PALOU, A. 2000. Opposite effects of feeding a vitamin A-deficient diet and retinoic acid treatment on brown adipose tissue uncoupling protein 1 (UCP1), UCP2 and leptin expression. *J Endocrinol,* 166**,** 511-7.

BONNET, M., BERNARD, L., BES, S. & LEROUX, C. 2013. Selection of reference genes for quantitative real-time PCR normalisation in adipose tissue, muscle, liver and mammary gland from ruminants. *Animal.* England.

BORG, K. E., LUNSTRA, D. D. & CHRISTENSON, R. K. 1993. Semen characteristics, testicular size, and reproductive hormone concentrations in mature Duroc, Meishan, Fengjing, and Minzhu boars. *Biol Reprod,* 49**,** 515-21.

BORZACCHIELLO, G. & ROPERTO, F. 2008. Bovine papillomaviruses, papillomas and cancer in cattle. *Vet Res,* 39**,** 45.

BOUSQUET, D., BOUCHARD, É. & DUTREMBLAY, D. Decreasing Fertility in Dairy Cows: Myth or Reality? *In:* CALLESEN, H., ed. 23rd World Buiatrics Congress, July 11-16, 2004 2004 Québec, Canada. World Buiatrics Congress.

BOWMANA, A., FROUDB, J., JOHALC, S., LAWD, J., LEAVERB, A. & WILLIAMS, K. 2012. Bringing home the bacon: from trader mentalities to indutrial policies. *CRESC Public Interest Report* Manchester, UK: Centre for Research on Socio-Cultural Change.

BOZAOGLU, K., CURRAN, J. E., STOCKER, C. J., ZAIBI, M. S., SEGAL, D., KONSTANTOPOULOS, N., MORRISON, S., CARLESS, M., DYER, T. D., COLE, S. A., GORING, H. H., MOSES, E. K., WALDER, K., CAWTHORNE, M. A., BLANGERO, J. & JOWETT, J. B. 2010. Chemerin, a novel adipokine in the regulation of angiogenesis. *J Clin Endocrinol Metab,* 95**,** 2476-85.

BPA. 2015. *Official policy to focus on a single type of pig* [Online]. Cambridge, UK: British Pig Association. Available: http://www.britishpigs.org.uk/trad3.htm [Accessed 31st January 2015 2015].

BPEX. 2015. *UK pig meat export* [Online]. Warwickshire, UK: Agriculture & Horticulture Development Board. Available: http://development.bpex.org.uk/prices-facts-figures/imports-exports/UKpigmeatexports.aspx [Accessed 24 August 2015].

BRADLEY, D. G., MACHUGH, D. E., CUNNINGHAM, P. & LOFTUS, R. T. 1996. Mitochondrial diversity and the origins of African and European cattle. *Proceedings of the National Academy of Sciences,* 93**,** 5131-5135.

BRANDEBOURG, T. D. & HU, C. Y. 2005. Regulation of differentiating pig preadipocytes by retinoic acid. *J Anim Sci.,* 83**,** 98-107.

BRAY, G. A. 1972. Lipogenesis in human adipose tissue: some effects of nibbling and gorging. *J Clin Invest,* 51**,** 537-48.

BREVIK, A., VEIEROD, M. B., DREVON, C. A. & ANDERSEN, L. F. 2005. Evaluation of the odd fatty acids 15:0 and 17:0 in serum and adipose tissue as markers of intake of milk and dairy fat. *Eur J Clin Nutr,* 59**,** 1417-1422.

BROOKS, M. J., RAJASIMHA, H. K., ROGER, J. E. & SWAROOP, A. 2011. Next-generation sequencing facilitates quantitative analysis of wild-type and Nrl(-/-) retinal transcriptomes. *Mol Vis,* 17**,** 3034-54.

BROWN, J. L. 2000. Reproductive endocrine monitoring of elephants: An essential tool for assisting captive management. *Zoo Biology,* 19**,** 347-367.

BROWN, J. L. 2014. Comparative Reproductive Biology of Elephants. *In:* HOLT, W. V., BROWN, J. L. & COMIZZOLI, P. (eds.) *Reproductive Sciences in Animal Conservation.* Springer New York.

BROWN, J. L., HILDEBRANDT, T. B., THEISON, W. & NEIFFER, D. L. 1999. Endocrine and ultrasound evaluation of a non-cycling African elephant: Identification of an ovarian follicular cyst. *Zoo Biology,* 18**,** 223-232.

BROWN, J. L., OLSON, D., KEELE, M. & FREEMAN, E. W. 2004. Survey of the reproductive cyclicity status of Asian and African elephants in North America. *Zoo Biology,* 23**,** 309-321.

BROWN, L. M. & CLEGG, D. J. 2010. Central effects of estradiol in the regulation of food intake, body weight, and adiposity. *The Journal of Steroid Biochemistry and Molecular Biology,* 122**,** 65-73.

BRÜGGEMANN, M., RITGEN, M., LADETTO, M., PEPIN, F., TRAUTMANN, H., CARLTON, V., HOELZER, D., GÖKBUGET, N., KNEBA, M., FAHAM, M. & POTT, C. 2013. Next-Generation Sequencing and Real-Time Quantitative PCR For Quantification Of Low-Level Minimal Residual Disease In Acute Lymphoblastic Leukemia Of Adults. *Blood,* 122**,** 351-351.

BUCCIONI, A., DECANDIA, M., MINIERI, S., MOLLE, G. & CABIDDU, A. 2012. Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. *Animal Feed Science and Technology,* 174**,** 1-25.

BUDAK, E., FERNANDEZ SANCHEZ, M., BELLVER, J., CERVERO, A., SIMON, C. & PELLICER, A. 2006. Interactions of the hormones leptin, ghrelin,

adiponectin, resistin, and PYY3-36 with the reproductive system. *Fertil Steril,* 85**,** 1563-81.

BUDHU, A. S. & NOY, N. 2002. Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid-induced growth arrest. *Mol Cell Biol,* 22**,** 2632-41.

BULUN, S. E. & ADASHI, E. Y. 2003. The physiology and pathology of the female reproductive axis. *In:* LARSEN, P. R. K., H.M.MELMED, S.POLONSKY, K.S. (ed.) *Williams Textbook of Endocrinology.* Philadelphia, Pennsylvania: Saunders.

BURGI, B., LICHTENSTEIGER, W., LAUBER, M. E. & SCHLUMPF, M. 1999. Ontogeny of diazepam binding inhibitor/acyl-CoA binding protein mRNA and peripheral benzodiazepine receptor mRNA expression in the rat. *J Neuroendocrinol,* 11**,** 85-100.

BURKS, D. J., FONT DE MORA, J., SCHUBERT, M., WITHERS, D. J., MYERS, M. G., TOWERY, H. H., ALTAMURO, S. L., FLINT, C. L. & WHITE, M. F. 2000. IRS-2 pathways integrate female reproduction and energy homeostasis. *Nature,* 407**,** 377-82.

BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., VANDESOMPELE, J. & WITTWER, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem,* 55**,** 611-22.

CAIN, J. M., ZAINO, R., SHEARER, D., BENNETT, R. A., OLT, G. & WEISZ, J. 2002. Expression of a retinol dehydrogenase (hRoDH-4), a member of the retinol/steroid dehydrogenase family implicated in retinoic acid biosynthesis, in normal and neoplastic endometria. *Am J Obstet Gynecol,* 186**,** 675-83.

CALPE, S., WANG, N., ROMERO, X., BERGER, S. B., LANYI, A., ENGEL, P. & TERHORST, C. 2008. The SLAM and SAP gene families control innate and adaptive immune responses. *Adv Immunol,* 97**,** 177-250.

CAMPBELL, R. G., TAVERNER, M. R. & CURIC, D. M. 1984. Effect of feeding level and dietary protein content on the growth, body composition and rate of protein deposition in pigs growing from 45 to 90 kg. *Animal Science,* 38**,** 233-240.

CAMPBELL, R. G., TAVERNER, M. R. & CURIC, D. M. 1985. The influence of feeding level on the protein requirement of pigs between 20 and 45 kg live weight. *Animal Science,* 40**,** 489-496.

CAMPFIELD, L. A., SMITH, F. J. & BURN, P. 1996. The OB protein (leptin) pathway--a link between adipose tissue mass and central neural networks. *Horm Metab Res,* 28**,** 619-32.

CAMPFIELD, L. A., SMITH, F. J., GUISEZ, Y., DEVOS, R. & BURN, P. 1995. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science,* 269**,** 546-9.

CANARIO, L., CANTONI, E., LE BIHAN, E., CARITEZ, J. C., BILLON, Y., BIDANEL, J. P. & FOULLEY, J. L. 2006. Between-breed variability of stillbirth and its relationship with sow and piglet characteristics. *Journal of Animal Science,* 84**,** 3185-3196.

CANNON, B. & NEDERGAARD, J. 2008. Developmental biology: Neither fat nor flesh. *Nature,* 454**,** 947-948.

CANNON, B. & NEDERGAARD, J. A. N. 2004. Brown adipose tissue: Function and physiological significance. *Physiological Reviews,* 84**,** 277-359.

CARLSON, B. A. 2012. Diversity matters: the importance of comparative studies and the potential for synergy between neuroscience and evolutionary biology. *Arch Neurol,* 69**,** 987-93.

CARSTAIRS, J. A., MORROW, D. A. & EMERY, R. S. 1980. Postpartum reproductive function of daity cows as influenced by energy and phosphorus status. *J Anim Sci,* 51**,** 1122-30.

CECAVA, M. J. 1995a. Protein requirements of beef cattle. *In:* PERRY, T. W. & CECAVA, M. J. (eds.) *Beef cattle feeding and nutrition.* 2nd ed. San Diego, California, USA: Academic Press Inc.

CECAVA, M. J. 1995b. Rumen Physiology and energy requirements. *In:* PERRY, T. W. & CECAVA, M. J. (eds.) *Beef cattle feeding and nutrition.* 2nd ed. San Diego, California, USA: Academic Press Inc.

CERVERO, A., DOMINGUEZ, F., HORCAJADAS, J. A., QUINONERO, A., PELLICER, A. & SIMON, C. 2006. The role of the leptin in reproduction. *Curr Opin Obstet Gynecol,* 18**,** 297-303.

CHAKAROUN, R., RASCHPICHLER, M., KLOTING, N., OBERBACH, A., FLEHMIG, G., KERN, M., SCHON, M. R., SHANG, E., LOHMANN, T., DRESSLER, M., FASSHAUER, M., STUMVOLL, M. & BLUHER, M. 2012. Effects of weight loss and exercise on chemerin serum concentrations and adipose tissue expression in human obesity. *Metabolism,* 61**,** 706-14.

CHAKRABARTI, J. 2013. Serum leptin level in women with polycystic ovary syndrome: correlation with adiposity, insulin, and circulating testosterone. *Ann Med Health Sci Res,* 3**,** 191-6.

CHAKRAVARTHY, M. V., PAN, Z., ZHU, Y., TORDJMAN, K., SCHNEIDER, J. G., COLEMAN, T., TURK, J. & SEMENKOVICH, C. F. 2005. "New" hepatic fat activates PPARalpha to maintain glucose, lipid, and cholesterol homeostasis. *Cell Metab,* 1**,** 309-22.

CHALDAKOV, G. N., FIORE, M., TONCHEV, A. B. & ALOE, L. 2010. Neuroadipology: a novel component of neuroendocrinology. *Cell Biol Int,* 34**,** 1051-3.

CHAN, J. & MANTZOROS, C. 2001. Leptin and the Hypothalamic-Pituitary Regulation of the Gonadotropin-Gonadal Axis. *Pituitary,* 4**,** 87-92.

CHAPMAN, S. J., KHOR, C. C., VANNBERG, F. O., RAUTANEN, A., SEGAL, S., MOORE, C. E., DAVIES, R. J., DAY, N. P., PESHU, N., CROOK, D. W., BERKLEY, J. A., WILLIAMS, T. N., SCOTT, J. A. & HILL, A. V. 2010. NFKBIZ polymorphisms and susceptibility to pneumococcal disease in European and African populations. *Genes Immun,* 11**,** 319-25.

CHARLTON, H. 2008. Hypothalamic Control of Anterior Pituitary Function: A History. *Journal of Neuroendocrinology,* 20**,** 641-646.

CHAWG 2014. GB Cattle Health & Welfare Group Second report: July 2014. Warwickshire: Cattle Health & Welfare Group.

CHAWLA, A., BARAK, Y., NAGY, L., LIAO, D., TONTONOZ, P. & EVANS, R. M. 2001. PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat Med,* 7**,** 48-52.

CHAWLA, A., SCHWARZ, E. J., DIMACULANGAN, D. D. & LAZAR, M. A. 1994. Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. *Endocrinology,* 135**,** 798-800.

CHELIKANI, P. K., GLIMM, D. R. & KENNELLY, J. J. 2003. Short Communication: Tissue Distribution of Leptin and Leptin Receptor mRNA in the Bovine. *Journal of Dairy Science,* 86**,** 2369-2372.

CHEMNITZ, C. 2014. The rise of the global market. *In:* CHEMNITZ, C., BECHEVA, S., BARTZ, D., MUNDY, P., SCHMIDT-LANDENBERGER , E., CORNELY, B. & MAHLKE, S. (eds.) *Meat Atlas.* Berlin, GermanyBrussel, Belgium Heinrich Böll FoundationFriends of the Earth Europe.

CHEN, D., ZHAO, M. & MUNDY, G. R. 2004. Bone morphogenetic proteins. *Growth Factors,* 22**,** 233-41.

CHEN, M. T., GUO, H. L., TSENG, T. F., ROAN, S. W. & NGAPO, T. M. 2010. Consumer choice of pork chops in Taiwan. *Meat Sci,* 85**,** 555-9.
CHIAN, R. C., AO, A., CLARKE, H. J., TULANDI, T. & TAN, S. L. 1999. Production of steroids from human cumulus cells treated with different concentrations of gonadotropins during culture in vitro. *Fertil Steril,* 71**,** 61-6.
CHILLIARD, Y., FERLAY, A. & DOREAU, M. 2001. Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livestock Production Science,* 70**,** 31-48.
CHIRALA, S. S. & WAKIL, S. J. 2004. Structure and function of animal fatty acid synthase. *Lipids,* 39**,** 1045-53.
CHOONG, S. S., FULTON, J., EMES, R. D., YON, L., HEERY, D. M. & MONGAN, N. P. 2015. Retinoids. *In:* CAPLAN, M.J. (ed.) *Reference Module in Biomedical Science: Cancer and Endocrine Diseases.* Elsevier.
CILDIR, G., AKINCILAR, S. C. & TERGAONKAR, V. 2013. Chronic adipose tissue inflammation: all immune cells on the stage. *Trends in Molecular Medicine,* 19**,** 487-500.
CINTI, S. 2001. The adipose organ: morphological perspectives of adipose tissues. *Proc Nutr Soc,* 60**,** 319-28.
CITES. 2013. *The CITES species* [Online]. Geneva, Switzerland: CITES. [Accessed 18 July 2015].
CITIRAK, G., WITTING, N., DUNO, M., WERLAUFF, U., PETRI, H. & VISSING, J. 2014. Frequency and phenotype of patients carrying TPM2 and TPM3 gene mutations in a cohort of 94 patients with congenital myopathy. *Neuromuscul Disord,* 24**,** 325-30.
CLARKE, I. J. & HENRY, B. A. 1999. Leptin and reproduction. *Rev Reprod,* 4**,** 48-55.
CLUBB, R. & MASON, G. 2002. A review of the welfare of zoo elephants in Europe. Horsham, UK: RSPCA.
COHEN, P. & FRIEDMAN, J. M. 2004. Leptin and the control of metabolism: role for stearoyl-CoA desaturase-1 (SCD-1). *J Nutr,* 134**,** 2455s-2463s.
COHEN-ZINDER, M., SEROUSSI, E., LARKIN, D. M., LOOR, J. J., EVERTS-VAN DER WIND, A., LEE, J. H., DRACKLEY, J. K., BAND, M. R., HERNANDEZ, A. G., SHANI, M., LEWIN, H. A., WELLER, J. I. & RON, M. 2005. Identification of a missense mutation in the bovine ABCG2 gene with a major effect on the QTL on chromosome 6 affecting milk yield and composition in Holstein cattle. *Genome Res,* 15**,** 936-44.
COLEMAN, D. L. 1973. Effects of parabiosis of obese with diabetes and normal mice. *Diabetologia,* 9**,** 294-298.
COLEMAN, D. L. 1978. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia,* 14**,** 141-8.
COLLINS, S. & SURWIT, R. S. 2001. The beta-adrenergic receptors and the control of adipose tissue metabolism and thermogenesis. *Recent Prog Horm Res,* 56**,** 309-28.
CONSIDINE, R. V., NYCE, M. R., MORALES, L. M., MAGOSIN, S. A., SINHA, M. K., BAUER, T. L., ROSATO, E. L., COLBERG, J. & CARO, J. F. 1996. Paracrine stimulation of preadipocyte-enriched cell cultures by mature adipocytes. *Am J Physiol,* 270**,** E895-9.
COOKE, R., LODGEA, G. A. & LEWIS, D. 1972. Influence of energy and protein concentration in the diet on the performance of growing pigs 1. Response to protein intake on a high-energy diet. *Animal Production,* 14**,** 35-46
COUSIN, B., CINTI, S., MORRONI, M., RAIMBAULT, S., RICQUIER, D., PENICAUD, L. & CASTEILLA, L. 1992. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J Cell Sci,* 103 (Pt 4)**,** 931-42.

CRANDALL, D. L., GOLDSTEIN, B. M., HUGGINS, F. & CERVONI, P. 1984. Adipocyte blood flow: influence of age, anatomic location, and dietary manipulation. *Am J Physiol,* 247**,** R46-51.

CROCINI, C., ARIMURA, T., REISCHMANN, S., EDER, A., BRAREN, I., HANSEN, A., ESCHENHAGEN, T., KIMURA, A. & CARRIER, L. 2013. Impact of ANKRD1 mutations associated with hypertrophic cardiomyopathy on contraction parameters of engineered heart tissue. *Basic Res Cardiol,* 108**,** 349.

CUI, Y., MIYOSHI, K., CLAUDIO, E., SIEBENLIST, U. K., GONZALEZ, F. J., FLAWS, J., WAGNER, K.-U. & HENNIGHAUSEN, L. 2002. Loss of the Peroxisome Proliferation-activated Receptor gamma (PPARγ) Does Not Affect Mammary Development and Propensity for Tumor Formation but Leads to Reduced Fertility. *Journal of Biological Chemistry,* 277**,** 17830-17835.

CUNNINGHAM, J. G. & KLEIN, B. G. 2007. Nutrient utilization during absorptive phase. *Textboook of Veterinary Physiology.* 4th ed. St Louis, Missouri: Saunders Elsevier.

D'SOUZA, D. N., PETHICK, D. W., DUNSHEA, F. R., PLUSKE, J. R. & MULLAN, B. P. 2003. Nutritional manipulation increases intramuscular fat levels in the *Longissimus* muscle of female finisher pigs. *Australian Journal of Agricultural Research,* 54**,** 745-749.

DAM, A. H., KOSCINSKI, I., KREMER, J. A., MOUTOU, C., JAEGER, A. S., OUDAKKER, A. R., TOURNAYE, H., CHARLET, N., LAGIER-TOURENNE, C., VAN BOKHOVEN, H. & VIVILLE, S. 2007. Homozygous mutation in SPATA16 is associated with male infertility in human globozoospermia. *Am J Hum Genet,* 81**,** 813-20.

DANCE, L. J. E., MATTHEWS, K. R. & DORAN, O. 2009. Effect of breed on fatty acid composition and stearoyl-CoA desaturase protein expression in the Semimembranosus muscle and subcutaneous adipose tissue of cattle. *Livestock Science,* 125**,** 291-297.

DANFÆR, A. & STRATHE, A. B. 2012. Quantitative and physiological aspects of pig growth *Nutritional physiology of the pig.* Copenhagen: Danish Pig Research Centre.

DANI, C., SMITH, A. G., DESSOLIN, S., LEROY, P., STACCINI, L., VILLAGEOIS, P., DARIMONT, C. & AILHAUD, G. 1997. Differentiation of embryonic stem cells into adipocytes in vitro. *J Cell Sci,* 110 (Pt 11)**,** 1279-85.

DAY, M. L., IMAKAWA, K., ZALESKY, D. D., KITTOK, R. J. & KINDER, J. E. 1986. Effects of restriction of dietary energy intake during the prepubertal period on secretion of luteinizing hormone and responsiveness of the pituitary to luteinizing hormone-releasing hormone in heifers. *J Anim Sci,* 62**,** 1641-8.

DE HAAN, W., BHATTACHARJEE, A., RUDDLE, P., KANG, M. H. & HAYDEN, M. R. 2014. ABCA1 in adipocytes regulates adipose tissue lipid content, glucose tolerance, and insulin sensitivity. *Journal of Lipid Research,* 55**,** 516-523.

DE HOON, M. J. L., IMOTO, S., NOLAN, J. & MIYANO, S. 2004. Open source clustering software. *Bioinformatics,* 20**,** 1453-1454.

DE LA RUA-DOMENECH, R. 2006. Human Mycobacterium bovis infection in the United Kingdom: Incidence, risks, control measures and review of the zoonotic aspects of bovine tuberculosis. *Tuberculosis (Edinb),* 86**,** 77-109.

DE SOUZA VALENTE DA SILVA, L., VALERIA DA VEIGA, G. & RAMALHO, R. A. 2007. Association of serum concentrations of retinol and carotenoids with overweight in children and adolescents. *Nutrition,* 23**,** 392-397.

DECSI, T., MOLNÁR, D. & KOLETZKO, B. 1997. Reduced plasma concentrations of alpha- tocopherol and beta-carotene in obese boys. *The Journal of Pediatrics,* 130**,** 653-655.

DEFRA 2014. UK - household purchases. *Family Food Datasets.* December 2014 ed. Lincoln, UK: Department for Environment, Food & Rural Affairs.

DEFRA 2015a. Agriculture in the United Kingdom data sets. London, UK: Department for Environment, Food & Rural Affairs.
DEFRA 2015b. Livestock. *Agriculture in the United Kingdom data sets.* Lincoln, UK: Department for Environment, Food & Rural Affairs.
DEFRA 2015c. Monthly publication of National Statistics on the Incidence of Tuberculosis (TB) in Cattle to end May 2015 for Great Britain. Lincoln, UK: Department for Environment Food and Rural Affairs.
DELVA, L., BASTIE, J. N., ROCHETTE-EGLY, C., KRAIBA, R., BALITRAND, N., DESPOUY, G., CHAMBON, P. & CHOMIENNE, C. 1999. Physical and functional interactions between cellular retinoic acid binding protein II and the retinoic acid-dependent nuclear complex. *Mol Cell Biol,* 19**,** 7158-67.
DESFORGES, M., PARSONS, L., WESTWOOD, M., SIBLEY, C. P. & GREENWOOD, S. L. 2013. Taurine transport in human placental trophoblast is important for regulation of cell differentiation and survival. *Cell Death Dis,* 4**,** e559.
DIBNER, C., SCHIBLER, U. & ALBRECHT, U. 2010. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol,* 72**,** 517-49.
DICKERSON, J. W., GRESHAM, G. A. & MCCANCE, R. A. 1964. The effect of undernutrition and rehabilitation on the development of the reproductive organs: pigs. *J Endocrinol,* 29**,** 111-8.
DILLON, P., BERRY, D. P., EVANS, R. D., BUCKLEY, F. & HORAN, B. 2006. Consequences of genetic selection for increased milk production in European seasonal pasture based systems of milk production. *Livestock Science,* 99**,** 141-158.
DIXON, J. B. 2010. Mechanisms of chylomicron uptake into lacteals. *Annals of the New York Academy of Sciences,* 1207**,** E52-E57.
DOMENE, H. M., HWA, V., JASPER, H. G. & ROSENFELD, R. G. 2011. Acid-labile subunit (ALS) deficiency. *Best Pract Res Clin Endocrinol Metab,* 25**,** 101-13.
DONG, D., RUUSKA, S. E., LEVINTHAL, D. J. & NOY, N. 1999. Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. *J Biol Chem,* 274**,** 23695-8.
DOOLITTLE, R. F. 1981. Similar amino acid sequences: chance or common ancestry? *Science,* 214**,** 149-59.
DOORNENBAL, H. & TONG, A. K. 1981. Growth, development and chemical composition of the pig. IV. Relative growth of visceral organs. *Growth,* 45**,** 279-85.
DOUGLAS-HAMILTON, I., BARNES, R. F. W., SHOSHANI, H., WILLIAMS, A. C. & JOHNSINGH, A. J. T. 2001. Elephants. *In:* MACDONALD, D. (ed.) *The New Encyclopedia of Mammals.* Oxford, UK: Oxford University Press.
DOW, T. L., HOLASKOVA, I. & BROWN, J. L. 2011. Results of the third reproductive assessment survey of North American Asian (Elephas maximus) and African (Loxodonta africana) female elephants. *Zoo Biol,* 30**,** 699-711.
DRANSFIELD, E., NGAPO, T. M., NIELSEN, N. A., BREDAHL, L., SJÖDÉN, P. O., MAGNUSSON, M., CAMPO, M. M. & NUTE, G. R. 2005. Consumer choice and suggested price for pork as influenced by its appearance, taste and information concerning country of origin and organic pig production. *Meat Science,* 69**,** 61-70.
DROLET, R., RICHARD, C., SNIDERMAN, A. D., MAILLOUX, J., FORTIER, M., HUOT, C., RHEAUME, C. & TCHERNOF, A. 2008. Hypertrophy and hyperplasia of abdominal adipose tissues in women. *Int J Obes (Lond),* 32**,** 283-91.
DUCROCQ, V., QUAAS, R. L., POLLAK, E. J. & CASELLA, G. 1998. Length of Productive Life of Dairy Cows. 2. Variance Component Estimation and Sire Evaluation. *Journal of Dairy Science,* 71**,** 3071-3079.

DUDEMAINE, P. L., THIBAULT, C., ALAIN, K. & BISSONNETTE, N. 2014. Genetic variations in the SPP1 promoter affect gene expression and the level of osteopontin secretion into bovine milk. *Anim Genet,* 45**,** 629-40.

DUIJVESTEIJN, N., KNOL, E. F., MERKS, J. W., CROOIJMANS, R. P., GROENEN, M. A., BOVENHUIS, H. & HARLIZIUS, B. 2010. A genome-wide association study on androstenone levels in pigs reveals a cluster of candidate genes on chromosome 6. *BMC Genet,* 11**,** 42.

DUMONT, J., PETRI, S., PELLEGRIN, F., TERRET, M. E., BOHNSACK, M. T., RASSINIER, P., GEORGET, V., KALAB, P., GRUSS, O. J. & VERLHAC, M. H. 2007. A centriole- and RanGTP-independent spindle assembly pathway in meiosis I of vertebrate oocytes. *J Cell Biol,* 176**,** 295-305.

DUNFORD, L., GARDNER, D., DUCKWORTH, B., O'SULLIVAN, S. & DEVONALD, M. 2014. The remote organ effects of acute kidney injury in a porcine model. *Endocrine Abstracts,* 34**,** 157.

DUNSHEA, F. R., D'SOUZA, D. N., PETHICK, D. W., HARPER, G. S. & WARNER, R. D. 2005. Effects of dietary factors and other metabolic modifiers on quality and nutritional value of meat. *Meat Science,* 71**,** 8-38.

EBERLE, D., HEGARTY, B., BOSSARD, P., FERRE, P. & FOUFELLE, F. 2004. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie,* 86**,** 839-48.

EBLEX 2008. *In the balance? The future of English beef industry.,* Warwickshire, UK, EBLEX.

EBLEX 2012. Balancing the market: Securing the future for English beef supply. Warwickshire, UK: EBLEX.

EBLEX 2015. The outlook of UK beef market: April 2015. Warwickshire, UK: Agriculture and Horticulture Development Board.

EDWARDS, C. J., BOLLONGINO, R., SCHEU, A., CHAMBERLAIN, A., TRESSET, A., VIGNE, J.-D., BAIRD, J. F., LARSON, G., HO, S. Y. W., HEUPINK, T. H., SHAPIRO, B., FREEMAN, A. R., THOMAS, M. G., ARBOGAST, R.-M., ARNDT, B., BARTOSIEWICZ, L., BENECKE, N., BUDJA, M., CHAIX, L., CHOYKE, A. M., COQUEUGNIOT, E., DÖHLE, H.-J., GÖLDNER, H., HARTZ, S., HELMER, D., HERZIG, B., HONGO, H., MASHKOUR, M., ÖZDOGAN, M., PUCHER, E., ROTH, G., SCHADE-LINDIG, S., SCHMÖLCKE, U., SCHULTING, R. J., STEPHAN, E., UERPMANN, H.-P., VÖRÖS, I., VOYTEK, B., BRADLEY, D. G. & BURGER, J. 2007. Mitochondrial DNA analysis shows a Near Eastern Neolithic origin for domestic cattle and no indication of domestication of European aurochs. *Proceedings of the Royal Society of London B: Biological Sciences,* 274**,** 1377-1385.

EDWARDS, C. J., GINJA, C., KANTANEN, J., PÉREZ-PARDAL, L., TRESSET, A., STOCK, F., GAMA, L. T., PENEDO, M. C. T., BRADLEY, D. G., LENSTRA, J. A., NIJMAN, I. J. & EUROPEAN CATTLE GENETIC DIVERSITY, C. 2011. Dual Origins of Dairy Cattle Farming – Evidence from a Comprehensive Survey of European Y-Chromosomal Variation. *PLoS ONE,* 6**,** e15922.

EGGERT, J. M., SHEISS, E. B., SCHINCKEL, A. P., FORREST, J. C., GRANT, A. L., MILLS, S. E. & WATKINS, B. A. 1996. Effects of genotype, sex, slaughter weight, and dietary fat on pig growth, carcass composition, and pig quality. *Swine Day Report* Indiana, USA: Purdue University.

EGGERT, L. S., RASNER, C. A. & WOODRUFF, D. S. 2002. The evolution and phylogeography of the African elephant inferred from mitochondrial DNA sequence and nuclear microsatellite markers. *Proceedings of the Royal Society of London B: Biological Sciences,* 269**,** 1993-2006.

EGUCHI, J., WANG, X., YU, S., KERSHAW, E. E., CHIU, P. C., DUSHAY, J., ESTALL, J. L., KLEIN, U., MARATOS-FLIER, E. & ROSEN, E. D. 2011. Transcriptional control of adipose lipid handling by IRF4. *Cell Metab,* 13**,** 249-59.

EGUCHI, J., YAN, Q. W., SCHONES, D. E., KAMAL, M., HSU, C. H., ZHANG, M. Q., CRAWFORD, G. E. & ROSEN, E. D. 2008. Interferon regulatory factors are transcriptional regulators of adipogenesis. *Cell Metab,* 7**,** 86-94.

EINSTEIN, F. H., FISHMAN, S., MUZUMDAR, R. H., YANG, X. M., ATZMON, G. & BARZILAI, N. 2008. Accretion of visceral fat and hepatic insulin resistance in pregnant rats. *Am J Physiol Endocrinol Metab,* 294**,** E451-5.

ELIZONDO, G., CORCHERO, J., STERNECK, E. & GONZALEZ, F. J. 2000. Feedback inhibition of the retinaldehyde dehydrogenase gene ALDH1 by retinoic acid through retinoic acid receptor alpha and CCAAT/enhancer-binding protein beta. *J Biol Chem,* 275**,** 39747-53.

ELTRINGHAM, S. K. 1982. Physiology and growth. *Elephants.* Dorset, UK: Blandford Press.

EMES, R. D. 2008. Inferring function from homology. *In:* KEITH, J. M. (ed.) *Bioinformatics, Volume II: Structure, function and application.* Queensland, Australia: Humana Press.

EMMANUEL, B. 1978. The relative contribution of propionate, and long-chain even-numbered fatty acids to the production of long-chain odd-numbered fatty acids in rumen bacteria. *Biochim Biophys Acta,* 528**,** 239-46.

ENCODE. 2011. Standards, Guidelines and Best Practices for RNA-Seq Available: http://genome.ucsc.edu/ENCODE/protocols/dataStandards/ENCODE_RNAseq_Standards_V1.0.pdf [Accessed 2 December 2014].

ENGEL, K., HÖHNE, W. & HÄBERLE, J. 2009. Mutations and polymorphisms in the human argininosuccinate synthetase (ASS1) gene. *Human Mutation,* 30**,** 300-307.

ENSEMBL. 2015a. *Cow assembly and gene annotation* [Online]. Hinxton, UK: Ensembl. [Accessed 12 June 2015 2015].

ENSEMBL. 2015b. *Elephant assembly and gene annotation* [Online]. Hinxton, UK: Ensembl. [Accessed 24 July 2015].

ENSEMBL. 2015c. *Pig assembly and gene annotation* [Online]. Ensembl. Available: http://www.ensembl.org/Sus_scrofa/Info/Annotation#assembly [Accessed 20 January 2015].

EPSTEIN, J. & BICHARD, M. 1984. Pig. *In:* MASON, I. L. (ed.) *Evolution of Domesticated Animals.* Longman.

ERIKSON, D. W., BURGHARDT, R. C., BAYLESS, K. J. & JOHNSON, G. A. 2009. Secreted phosphoprotein 1 (SPP1, osteopontin) binds to integrin alpha v beta 6 on porcine trophectoderm cells and integrin alpha v beta 3 on uterine luminal epithelial cells, and promotes trophectoderm cell adhesion and migration. *Biol Reprod,* 81**,** 814-25.

ERNST, M. C., HAIDL, I. D., ZUNIGA, L. A., DRANSE, H. J., ROURKE, J. L., ZABEL, B. A., BUTCHER, E. C. & SINAL, C. J. 2012. Disruption of the chemokine-like receptor-1 (CMKLR1) gene is associated with reduced adiposity and glucose intolerance. *Endocrinology,* 153**,** 672-82.

ESTERBAUER, H., OBERKOFLER, H., KREMPLER, F. & PATSCH, W. 1999. Human peroxisome proliferator activated receptor gamma coactivator 1 (PPARGC1) gene: cDNA sequence, genomic organization, chromosomal localization, and tissue expression. *Genomics,* 62**,** 98-102.

EVANS, K. E. & HARRIS, S. 2008. Adolescence in male African elephants, Loxodonta africana, and the importance of sociality. *Animal Behaviour,* 76**,** 779-787.

EWAN, R. C. 1991. Energy utilization in swine nutrition. *In:* MILLER, E. R., ULLREY, D. E. & LEWIS, A. J. (eds.) *Swine Nutrition.* MA, USA: Butterworth-Heinemann.

FAILLACE, L. S. & HUNTER, M. G. 1994. Follicle development and oocyte maturation during the immediate preovulatory period in Meishan and white hybrid gilts. *J Reprod Fertil,* 101**,** 571-6.

FAINBERG, H. P., BODLEY, K., BACARDIT, J., LI, D., WESSELY, F., MONGAN, N. P., SYMONDS, M. E., CLARKE, L. & MOSTYN, A. 2012. Reduced neonatal mortality in Meishan piglets: a role for hepatic fatty acids? *PLoS One,* 7**,** e49101.

FANG, K., LI, H. F., HSIEH, C. H., LI, D. Y., SONG, D. C., CHENG, W. T. & GUO, Z. X. 2013. Differential autophagic cell death under stress with ectopic cytoplasmic and mitochondrial-specific PPP2R2B in human neuroblastoma cells. *Apoptosis,* 18**,** 627-38.

FAO. 2014. *Sources of meat* [Online]. Food and Agriculture Organisation of the United Nations. Available: http://www.fao.org/ag/againfo/themes/en/meat/backgr_sources.html [Accessed 26 October 2014 2014].

FARMER, S. R. 2006. Transcriptional control of adipocyte formation. *Cell Metab,* 4**,** 263-73.

FATIMA, S. S., REHMAN, R., BAIG, M. & KHAN, T. A. 2014. New roles of the multidimensional adipokine: Chemerin. *Peptides,* 62**,** 15-20.

FAUST, I. M., JOHNSON, P. R., STERN, J. S. & HIRSCH, J. 1978. Diet-induced adipocyte number increase in adult rats: a new model of obesity. *Am J Physiol,* 235**,** E279-86.

FDF 2012. *UK Food & Drink Export Performance Full Year 2011,* London, UK, Food and Drink Federation.

FDF. 2015. *UK food and drink export statistics for 2014* [Online]. London, UK: Food and Drink Federation. [Accessed 1 June 2015 2015].

FENTON, F. R., SCHWARTZ, F. L., BAZER, F. W., ROBISON, O. W. & ULBERG, L. C. 1972. Stage of gestation when uterine capacity limits embryo survival in gilts. *Journal of Animal Science,* 35**,** 383-388.

FERNANDEZ-RODRIGUEZ, A., MUNOZ, M., FERNANDEZ, A., PENA, R. N., TOMAS, A., NOGUERA, J. L., OVILO, C. & FERNANDEZ, A. I. 2011. Differential gene expression in ovaries of pregnant pigs with high and low prolificacy levels and identification of candidate genes for litter size. *Biol Reprod,* 84**,** 299-307.

FERNYHOUGH, M. E., OKINE, E., HAUSMAN, G., VIERCK, J. L. & DODSON, M. V. 2007. PPARgamma and GLUT-4 expression as developmental regulators/markers for preadipocyte differentiation into an adipocyte. *Domest Anim Endocrinol,* 33**,** 367-78.

FISLER, J. S., EGAWA, M. & BRAY, G. A. 1995. Peripheral 3-hydroxybutyrate and food intake in a model of dietary-fat induced obesity: effect of vagotomy. *Physiol Behav,* 58**,** 1-7.

FITCH, W. M. 2000. Homology a personal view on some of the problems. *Trends Genet,* 16**,** 227-31.

FLEURY, C., NEVEROVA, M., COLLINS, S., RAIMBAULT, S., CHAMPIGNY, O., LEVI-MEYRUEIS, C., BOUILLAUD, F., SELDIN, M. F., SURWIT, R. S., RICQUIER, D. & WARDEN, C. H. 1997. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat Genet,* 15**,** 269-72.

FLICEK, P., AHMED, I., AMODE, M. R., BARRELL, D., BEAL, K., BRENT, S., CARVALHO-SILVA, D., CLAPHAM, P., COATES, G., FAIRLEY, S., FITZGERALD, S., GIL, L., GARCÍA-GIRÓN, C., GORDON, L., HOURLIER, T., HUNT, S., JUETTEMANN, T., KÄHÄRI, A. K., KEENAN, S., KOMOROWSKA, M., KULESHA, E., LONGDEN, I., MAUREL, T., MCLAREN, W. M., MUFFATO, M., NAG, R., OVERDUIN, B., PIGNATELLI, M., PRITCHARD, B., PRITCHARD, E., RIAT, H. S., RITCHIE, G. R. S., RUFFIER, M., SCHUSTER, M., SHEPPARD, D., SOBRAL, D., TAYLOR, K., THORMANN, A., TREVANION, S., WHITE, S., WILDER, S. P., AKEN, B. L., BIRNEY, E., CUNNINGHAM, F., DUNHAM, I., HARROW, J., HERRERO, J., HUBBARD, T. J. P., JOHNSON, N., KINSELLA, R., PARKER, A., SPUDICH,

G., YATES, A., ZADISSA, A. & SEARLE, S. M. J. 2013. Ensembl 2013. *Nucleic Acids Research,* 41**,** D48-D55.
FORTOMARIS, P., ARSENOS, G., GEORGIADIS, M., BANOS, G., STAMATARIS, C. & ZYGOYIANNIS, D. 2006. Effect of meat appearance on consumer preferences for pork chops in Greece and Cyprus. *Meat Science,* 72**,** 688-696.
FOSTER, G. E., NAKANO, J., SHEEL, A. W., SIMPSON, J. A., ROAD, J. D. & REID, W. D. 2012. Serum skeletal troponin I following inspiratory threshold loading in healthy young and middle-aged men. *Eur J Appl Physiol,* 112**,** 3547-58.
FRANCIS, A., SUNITHA, B., VINODH, K., POLAVARAPU, K., KATKAM, S. K., MODI, S., BHARATH, M. M., GAYATHRI, N., NALINI, A. & THANGARAJ, K. 2014. Novel TCAP mutation c.32C>A causing limb girdle muscular dystrophy 2G. *PLoS One,* 9**,** e102763.
FRANKS, S., GILLING-SMITH, C., WATSON, H. & WILLIS, D. 1999. Insulin action in the normal and polycystic ovary. *Endocrinol Metab Clin North Am,* 28**,** 361-78.
FREEMAN, E. W., GUAGNANO, G., OLSON, D., KEELE, M. & BROWN, J. L. 2009. Social factors influence ovarian acyclicity in captive African elephants (Loxodonta africana). *Zoo Biol,* 28**,** 1-15.
FREEMAN, E. W., MEYER, J. M., PUTMAN, S. B., SCHULTE, B. A. & BROWN, J. L. 2013. Ovarian cycle activity varies with respect to age and social status in free-ranging elephants in Addo Elephant National Park, South Africa. *Conservation Physiology,* 1.
FREYTAG, S. O., PAIELLI, D. L. & GILBERT, J. D. 1994. Ectopic expression of the CCAAT/enhancer-binding protein alpha promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev,* 8**,** 1654-63.
FRIEDMAN, J. M. & HALAAS, J. L. 1998. Leptin and the regulation of body weight in mammals. *Nature,* 395**,** 763-70.
FRIEND, D. W. 1977. Effect of dietary energy and protein on age and weight at puberty of gilts. *J. Anim. Sci.,* 44 601-607.
FRIEND, D. W., LODGE, G. A. & ELLIOT, J. I. 1981. Effect of energy and dry matter intake on age, body weight and backfat at puberty and on embryo mortality in gilts. *J. Anim. Sci.,* 53**,** 118-124.
FRISCH, R. E. 1984. Body fat, puberty and fertility. *Biol Rev Camb Philos Soc,* 59**,** 161-88.
FRISCH, R. E. 1987. Body fat, menarche, fitness and fertility. *Hum Reprod,* 2**,** 521-33.
FRISCH, R. E. & MCARTHUR, J. W. 1974. Menstrual cycles: fatness as a determinant of minimum weight for height necessary for their maintenance or onset. *Science,* 185**,** 949-51.
FROMENT, P., FABRE, S., DUPONT, J., PISSELET, C., CHESNEAU, D., STAELS, B. & MONGET, P. 2003. Expression and functional role of peroxisome proliferator-activated receptor-gamma in ovarian folliculogenesis in the sheep. *Biol Reprod,* 69**,** 1665-74.
FROMENT, P., GIZARD, F., DEFEVER, D., STAELS, B., DUPONT, J. & MONGET, P. 2006. Peroxisome proliferator-activated receptors in reproductive tissues: from gametogenesis to parturition. *J Endocrinol,* 189**,** 199-209.
FROMENT, P. & TOURAINE, P. 2006. Thiazolidinediones and Fertility in Polycystic Ovary Syndrome (PCOS). *PPAR Research,* 2006**,** 73986.
FRUHBECK, G. 2001. A heliocentric view of leptin. *Proc Nutr Soc,* 60**,** 301-18.
GADEN, B. 2003. Growth, maturity and carcase specifications. NSW, Australia: Armidale Feeeder Steer School, NSW Agriculture.
GALIC, S., OAKHILL, J. S. & STEINBERG, G. R. 2010. Adipose tissue as an endocrine organ. *Molecular and Cellular Endocrinology,* 316**,** 129-139.

GAO, H., MEJHERT, N., FRETZ, J. A., ARNER, E., LORENTE-CEBRIAN, S., EHRLUND, A., DAHLMAN-WRIGHT, K., GONG, X., STROMBLAD, S., DOUAGI, I., LAURENCIKIENE, J., DAHLMAN, I., DAUB, C. O., RYDEN, M., HOROWITZ, M. C. & ARNER, P. 2014. Early B cell factor 1 regulates adipocyte morphology and lipolysis in white adipose tissue. *Cell Metab,* 19**,** 981-92.

GAO, Q. & HORVATH, T. L. 2008. Cross-talk between estrogen and leptin signaling in the hypothalamus. *American Journal of Physiology - Endocrinology and Metabolism,* 294**,** E817-E826.

GARCIA, M. R., AMSTALDEN, M., WILLIAMS, S. W., STANKO, R. L., MORRISON, C. D., KEISLER, D. H., NIZIELSKI, S. E. & WILLIAMS, G. L. 2002. Serum leptin and its adipose gene expression during pubertal development, the estrous cycle, and different seasons in cattle. *J Anim Sci,* 80**,** 2158-67.

GARCIA-CASTRO, B., ALVAREZ-ZAVALA, M., RIVEROS-MAGANA, A. R., ORTIZ-LAZARENO, P. C., RATKOVICH-GONZALEZ, S., HERNANDEZ-FLORES, G., BRAVO-CUELLAR, A., JAVE-SUAREZ, L. F. & AGUILAR-LEMARROY, A. 2013. Restoration of WNT4 inhibits cell growth in leukemia-derived cell lines. *BMC Cancer,* 13**,** 557.

GARCIA-GALIANO, D., ALLEN, S. J. & ELIAS, C. F. 2014. Role of the adipocyte-derived hormone leptin in reproductive control. *Horm Mol Biol Clin Investig,* 19**,** 141-9.

GARENC, C., PERUSSE, L., CHAGNON, Y. C., RANKINEN, T., GAGNON, J., BORECKI, I. B., LEON, A. S., SKINNER, J. S., WILMORE, J. H., RAO, D. C. & BOUCHARD, C. 2002. The alpha 2-adrenergic receptor gene and body fat content and distribution: the HERITAGE Family Study. *Mol Med,* 8**,** 88-94.

GARLAND, T., BENNETT, A. F. & REZENDE, E. L. 2005. Phylogenetic approaches in comparative physiology. *Journal of Experimental Biology,* 208**,** 3015-3035.

GAUB, M. P., LUTZ, Y., GHYSELINCK, N. B., SCHEUER, I., PFISTER, V., CHAMBON, P. & ROCHETTE-EGLY, C. 1998. Nuclear detection of cellular retinoic acid binding proteins I and II with new antibodies. *J Histochem Cytochem,* 46**,** 1103-11.

GBURCIK, V., CLEASBY, M. E. & TIMMONS, J. A. 2013. Loss of neuronatin promotes "browning" of primary mouse adipocytes while reducing Glut1-mediated glucose disposal. *Am J Physiol Endocrinol Metab.* United States.

GESTA, S., BLUHER, M., YAMAMOTO, Y., NORRIS, A. W., BERNDT, J., KRALISCH, S., BOUCHER, J., LEWIS, C. & KAHN, C. R. 2006. Evidence for a role of developmental genes in the origin of obesity and body fat distribution. *Proc Natl Acad Sci U S A,* 103**,** 6676-81.

GESTA, S., TSENG, Y. H. & KAHN, C. R. 2007. Developmental origin of fat: tracking obesity to its source. *Cell,* 131**,** 242-56.

GFS. 2015. *The food security problem* [Online]. Swindon: Biotechnology and Biological Sciences Research Council. [Accessed 5 September 2015 2015].

GIMENO, R. E., DEMBSKI, M., WENG, X., DENG, N., SHYJAN, A. W., GIMENO, C. J., IRIS, F., ELLIS, S. J., WOOLF, E. A. & TARTAGLIA, L. A. 1997. Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis. *Diabetes,* 46**,** 900-6.

GIUFFRA, E., KIJAS, J. M. H., AMARGER, V., CARLBORG, Ö., JEON, J. T. & ANDERSSON, L. 2000. The Origin of the Domestic Pig: Independent Domestication and Subsequent Introgression. *Genetics,* 154**,** 1785-1791.

GLATZ, J. F., LUIKEN, J. J. & BONEN, A. 2001. Involvement of membrane-associated proteins in the acute regulation of cellular fatty acid uptake. *J Mol Neurosci,* 16**,** 123-32; discussion 151-7.

GOLDBERG, I. J., ECKEL, R. H. & ABUMRAD, N. A. 2009. Regulation of fatty acid uptake into tissues: Lipoprotein lipase- And CD36-mediated pathways. *Journal of Lipid Research,* 50**,** S86-S90.

GORALSKI, K. B., MCCARTHY, T. C., HANNIMAN, E. A., ZABEL, B. A., BUTCHER, E. C., PARLEE, S. D., MURUGANANDAN, S. & SINAL, C. J. 2007. Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *J Biol Chem,* 282**,** 28175-28188.

GORALSKI, K. B. & SINAL, C. J. 2009. Elucidation of chemerin and chemokine-like receptor-1 function in adipocytes by adenoviral-mediated shRNA knockdown of gene expression. *Methods Enzymol,* 460**,** 289-312.

GORKIN, D. U. & REN, B. 2014. Genetics: Closing the distance on obesity culprits. *Nature,* 507**,** 309-10.

GRAVES, R. A., TONTONOZ, P. & SPIEGELMAN, B. M. 1992. Analysis of a tissue-specific enhancer: ARF6 regulates adipogenic gene expression. *Molecular and Cellular Biology,* 12**,** 1202-1208.

GREENE, S. A., SMITH, M. A., CARTWRIGHT, B. & BAUM, J. D. 1983. Comparison of human versus porcine insulin in treatment of diabetes in children. *Br Med J (Clin Res Ed),* 287**,** 1578-9.

GREENWAY, S. C., MCLEOD, R., HUME, S., ROSLIN, N. M., ALVAREZ, N., GIUFFRE, M., ZHAN, S. H., SHEN, Y., PREUSS, C., ANDELFINGER, G., JONES, S. J. & GERULL, B. 2014. Exome sequencing identifies a novel variant in ACTC1 associated with familial atrial septal defect. *Can J Cardiol,* 30**,** 181-7.

GREGORASZCZUK, E. L., PTAK, A., WOJCIECHOWICZ, T. & NOWAK, K. 2007. Action of IGF-I on expression of the long form of the leptin receptor (ObRb) in the prepubertal period and throughout the estrous cycle in the mature pig ovary. *J Reprod Dev,* 53**,** 289-95.

GREGORASZCZUK, E. L., WOJTOWICZ, A. K., PTAK, A. & NOWAK, K. 2003. In vitro effect of leptin on steroids' secretion by FSH- and LH-treated porcine small, medium and large preovulatory follicles. *Reprod Biol,* 3**,** 227-39.

GREGORASZCZUK, E. Ł. & RAK-MARDYŁA, A. 2013. Supraphysiological leptin levels shift the profile of steroidogenesis in porcine ovarian follicles toward progesterone and testosterone secretion through increased expressions of CYP11A1 and 17β-HSD: a tissue culture approach. *Reproduction,* 145**,** 311-317.

GRIMALDI, B., BELLET, M. M., KATADA, S., ASTARITA, G., HIRAYAMA, J., AMIN, R. H., GRANNEMAN, J. G., PIOMELLI, D., LEFF, T. & SASSONE-CORSI, P. 2010. PER2 controls lipid metabolism by direct regulation of PPARgamma. *Cell Metab,* 12**,** 509-20.

GRUMMER, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. *Journal of Dairy Science,* 76**,** 3882-3896.

GRUMMER, R. R. 2007. Strategies to improve fertility of high yielding dairy farms: Management of the dry period. *Theriogenology,* 68, Supplement 1**,** S281-S288.

GUILLOU, H., ZADRAVEC, D., MARTIN, P. G. P. & JACOBSSON, A. 2010. The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. *Progress in Lipid Research,* 49**,** 186-199.

GUSEV, Y. 2008. Computational methods for analysis of cellular functions and pathways collectively targeted by differentially expressed microRNA. *Methods,* 44**,** 61-72.

GUYTON, A. C. & HALL, J. E. 2006. Lipid metabolism. *Textbook of Medical Physiology.* 11th ed. Philadelphia, Pennsylvania: Elseview Saunders.

HAAPA-PAANANEN, S., KIVILUOTO, S., WALTARI, M., PUPUTTI, M., MPINDI, J. P., KOHONEN, P., TYNNINEN, O., HAAPASALO, H., JOENSUU, H., PERALA, M. & KALLIONIEMI, O. 2012. HES6 gene is selectively overexpressed in glioma and represents an important transcriptional regulator of glioma proliferation. *Oncogene,* 31**,** 1299-310.

HALESTRAP, A. P. & MEREDITH, D. 2004. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch,* 447**,** 619-28.

HALEY, C. S., LEE, G. J. & RITCHIE, M. 1995. Comparative reproductive performance in Meishan and Large White pigs and their crosses. *Animal Science,* 60**,** 259-267.

HAMPTON, M., MELVIN, R. G. & ANDREWS, M. T. 2013. Transcriptomic Analysis of Brown Adipose Tissue across the Physiological Extremes of Natural Hibernation. *PLoS ONE,* 8**,** e85157.

HAMZA, M. S., POTT, S., VEGA, V. B., THOMSEN, J. S., KANDHADAYAR, G. S., NG, P. W. P., CHIU, K. P., PETTERSSON, S., WEI, C. L., RUAN, Y. & LIU, E. T. 2009. *De-Novo* identification of PPARγ/RXR binding sites and direct targets during adipogenesis. *PLoS ONE,* 4**,** e4907.

HARMS, M. & SEALE, P. 2013. Brown and beige fat: development, function and therapeutic potential. *Nat Med,* 19**,** 1252-1263.

HARRIS, R. B. S. 2013. Leptin-induced increase in body fat content of rats. *American Journal of Physiology - Endocrinology and Metabolism,* 304**,** E267-E281.

HATT, J. M. & CLAUSS, M. 2006. Feeding Asian and African elephants Elephas maximus and Loxodonta africana in captivity. *International Zoo Yearbook,* 40**,** 88-95.

HAUNER, H., ENTENMANN, G., WABITSCH, M., GAILLARD, D., AILHAUD, G., NEGREL, R. & PFEIFFER, E. F. 1989. Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J Clin Invest,* 84**,** 1663-70.

HAUSER, N., MOUROT, J., DE CLERCQ, L., GENART, C. & REMACLE, C. 1997. The cellularity of developing adipose tissues in Pietrain and Meishan pigs. *Reproduction Nutrition Development,* 37**,** 617-625

HAUSMAN, D. B., DIGIROLAMO, M., BARTNESS, T. J., HAUSMAN, G. J. & MARTIN, R. J. 2001. The biology of white adipocyte proliferation. *Obes Rev,* 2**,** 239-54.

HAUSMAN, G. J. 1985. Cellular and Enzyme-Histochemical Aspects of Adipose Tissue Development in Obese (Ossabaw) and Lean (Crossbred) Pig Fetuses: An Ontogeny Study. *Journal of Animal Science,* 60**,** 1539-1552.

HAUSMAN, G. J. & BARB, C. R. 2010. Adipose tissue and the reproductive axis: biological aspects. *Endocr Dev,* 19**,** 31-44.

HAUSMAN, G. J., BARB, C. R. & LENTS, C. A. 2012. Leptin and reproductive function. *Biochimie,* 94**,** 2075-81.

HAUSMAN, G. J. & RICHARDSON, L. R. 1982. Histochemical and ultrastructural analysis of developing adipocytes in the fetal pig. *Acta Anat (Basel),* 114**,** 228-47.

HE, L., KIM, T., LONG, Q., LIU, J., WANG, P., ZHOU, Y., DING, Y., PRASAIN, J., WOOD, P. A. & YANG, Q. 2012. Carnitine Palmitoyltransferase-1b Deficiency Aggravates Pressure Overload–Induced Cardiac Hypertrophy Caused by Lipotoxicity. *Circulation,* 126**,** 1705-1716.

HELLERSTEIN, M. K. 2001. No common energy currency: de novo lipogenesis as the road less traveled. *The American Journal of Clinical Nutrition,* 74**,** 707-708.

HELLMER, J., MARCUS, C., SONNENFELD, T. & ARNER, P. 1992. Mechanisms for differences in lipolysis between human subcutaneous and omental fat cells. *J Clin Endocrinol Metab,* 75**,** 15-20.

HENRIKSON, R. C., KAYE, G. & MAZURKIEWICZ, J. E. 1999. Adipose Tissue. *In:* NIEGINSKI, E. A. (ed.) *NMS Histology (National Medical Series for Independent Study).* 3rd Revised edition ed. Baltimore, Maryland, USA: Lippincott Williams and Wilkins.

HERMES, R., OLSON, D., GÖRITZ, F., BROWN, J. L., SCHMITT, D. L., HAGAN, D., PETERSON, J. S., FRITSCH, G. & HILDEBRANDT, T. B. 2000. Ultrasonography of the estrous cycle in female African elephants (Loxodonta africana). *Zoo Biology,* 19**,** 369-382.

HERNANDEZ, S. C., FINLAYSON, H. A., ASHWORTH, C. J., HALEY, C. S. & ARCHIBALD, A. L. 2014. A genome-wide linkage analysis for reproductive traits in F2 Large White x Meishan cross gilts. *Anim Genet,* 45**,** 191-7.

HERNANDEZ, S. C., HOGG, C. O., BILLON, Y., SANCHEZ, M.-P., BIDANEL, J.-P., HALEY, C. S., ARCHIBALD, A. L. & ASHWORTH, C. J. 2013. Secreted Phosphoprotein 1 Expression in Endometrium and Placental Tissues of Hyperprolific Large White and Meishan Gilts. *Biology of Reproduction,* 88**,** 120.

HILDEBRANDT, T., GÖRITZ, F. & HERMES, R. 2006a. Ultrasonography: an important tool in captive breeding management in elephants and rhinoceroses. *European Journal of Wildlife Research,* 52**,** 23-27.

HILDEBRANDT, T. B., GORITZ, F., HERMES, R., SCHMITT, D. L., BROWN, J. L., SCHWAMMER, H., LOSKUTOFF, N., PRATT, N. C., LEHNHARDT, J. L., MONTALI, R. J. & OLSON, D. Artificial insemination of African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants American Association Zoo Veterinarians, 1999 Columbus, OH, USA., 83–86.

HILDEBRANDT, T. B., GÖRITZ, F., HERMES, R., REID, C., DEHNHARD, M. & BROWN, J. L. 2006b. Aspects of the reproductive biology and breeding management of Asian and African elephants Elephas maximus and Loxodonta africana. *International Zoo Yearbook,* 40**,** 20-40.

HIRSCH, J. & BATCHELOR, B. 1976. Adipose tissue cellularity in human obesity. *Clin Endocrinol Metab,* 5**,** 299-311.

HOCQUETTE, J. F. & BAUCHART, D. 1999. Intestinal absorption, blood transport and hepatic and muscle metabolism of fatty acids in preruminant and ruminant animals. *Reprod Nutr Dev,* 39**,** 27-48.

HOFFMANN, C., MOREAU, F., MOES, M., LUTHOLD, C., DIETERLE, M., GORETTI, E., NEUMANN, K., STEINMETZ, A. & THOMAS, C. 2014. Human muscle LIM protein dimerizes along the actin cytoskeleton and cross-links actin filaments. *Mol Cell Biol,* 34**,** 3053-65.

HOFMANN, C., LORENZ, K., BRAITHWAITE, S. S., COLCA, J. R., PALAZUK, B. J., HOTAMISLIGIL, G. S. & SPIEGELMAN, B. M. 1994. Altered gene expression for tumor necrosis factor-alpha and its receptors during drug and dietary modulation of insulin resistance. *Endocrinology,* 134**,** 264-70.

HOLLENBERG, A. N., SUSULIC, V. S., MADURA, J. P., ZHANG, B., MOLLER, D. E., TONTONOZ, P., SARRAF, P., SPIEGELMAN, B. M. & LOWELL, B. B. 1997. Functional antagonism between CCAAT/Enhancer binding protein-alpha and peroxisome proliferator-activated receptor-gamma on the leptin promoter. *J Biol Chem,* 272**,** 5283-90.

HOLLISTER-SMITH, J. A., POOLE, J. H., ARCHIE, E. A., VANCE, E. A., GEORGIADIS, N. J., MOSS, C. J. & ALBERTS, S. C. 2007. Age, musth and paternity success in wild male African elephants, Loxodonta africana. *Animal Behaviour,* 74**,** 287-296.

HOLLUNG, K., RISE, C. P., DREVON, C. A. & RESELAND, J. E. 2004. Tissue-specific regulation of leptin expression and secretion by all-trans retinoic acid. *J Cell Biochem,* 92**,** 307-15.

HOLLÓ, G., NUERNBERG, K., SOMOGYI, T., ANTON, I. & HOLLÓ, I. 2012. Comparison of fattening performance and slaughter value of local Hungarian cattle breeds to international breeds. *Archiv Tierzucht,* 55**,** 1-12.

HOLSTEIN-UK. 2014. *Education - Dairy cows* [Online]. Herts, UK: Holstein UK. [Accessed 12 June 2015 2015].

HOLT, I., JACQUEMIN, V., FARDAEI, M., SEWRY, C. A., BUTLER-BROWNE, G. S., FURLING, D., BROOK, J. D. & MORRIS, G. E. 2009. Muscleblind-like proteins: similarities and differences in normal and myotonic dystrophy muscle. *Am J Pathol,* 174**,** 216-27.

HOMKO, C. J., SIVAN, E., REECE, E. A. & BODEN, G. 1999. Fuel metabolism during pregnancy. *Semin Reprod Endocrinol,* 17**,** 119-25.

HOOD, R. L. & ALLEN, C. E. 1977. Cellularity of porcine adipose tissue: effects of growth and adiposity. *J Lipid Res,* 18**,** 275-84.

HORLICK, M. B., ROSENBAUM, M., NICOLSON, M., LEVINE, L. S., FEDUN, B., WANG, J., PIERSON, R. N., JR. & LEIBEL, R. L. 2000. Effect of puberty on the relationship between circulating leptin and body composition. *J Clin Endocrinol Metab,* 85**,** 2509-18.

HOUSEKNECHT, K. L. & PORTOCARRERO, C. P. 1998. Leptin and its receptors: regulators of whole-body energy homeostasis. *Domest Anim Endocrinol,* 15**,** 457-75.

HOVING, L. L., SOEDE, N. M., FEITSMA, H. & KEMP, B. 2012. Lactation weight loss in primiparous sows: consequences for embryo survival and progesterone and relations with metabolic profiles. *Reprod Domest Anim,* 47**,** 1009-16.

HSIAO, P.-W., LIN, D.-L., NAKAO, R. & CHANG, C. 1999. The Linkage of Kennedy's Neuron Disease to ARA24, the First Identified Androgen Receptor Polyglutamine Region-associated Coactivator. *Journal of Biological Chemistry,* 274**,** 20229-20234.

HU, E., TONTONOZ, P. & SPIEGELMAN, B. M. 1995. Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc Natl Acad Sci U S A,* 92**,** 9856-60.

HU, Y., YU, H., PASK, A. J., O'BRIEN, D. A., SHAW, G. & RENFREE, M. B. 2009. A-kinase anchoring protein 4 has a conserved role in mammalian spermatogenesis. *Reproduction,* 137**,** 645-653.

HUANG, D. W., SHERMAN, B. T. & LEMPICKI, R. A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc,* 4**,** 44-57.

HUNDLEY, A. F., YUAN, L. & VISCO, A. G. 2006. Skeletal muscle heavy-chain polypeptide 3 and myosin binding protein H in the pubococcygeus muscle in patients with and without pelvic organ prolapse. *Am J Obstet Gynecol,* 194**,** 1404-10.

HUNTER, M. G., BIGGS, C., FOXCROFT, G. R., MCNEILLY, A. S. & TILTON, J. E. 1993. Comparisons of endocrinology and behavioural events during the periovulatory period in Meishan and large-white hybrid gilts. *J Reprod Fertil,* 97**,** 475-80.

HUNTER, M. G. & PICTON, H. M. 1995. Effect of hCG administration at the onset of oestrus on early embryo survival and development in Meishan gilts. *Animal Reproduction Science,* 38**,** 231-238.

HUTCHINS, M. & CONWAY, W. G. 1995. Beyond Noah's Ark: the evolving role of modern zoological parks and aquariums in field conservation. *International Zoo Yearbook,* 34**,** 117-130.

IMAI, T., JIANG, M., CHAMBON, P. & METZGER, D. 2001. Impaired adipogenesis and lipolysis in the mouse upon selective ablation of the retinoid X receptor alpha mediated by a tamoxifen-inducible chimeric Cre recombinase (Cre-ERT2) in adipocytes. *Proc Natl Acad Sci U S A,* 98**,** 224-8.

IMAI, T., TAKAKUWA, R., MARCHAND, S., DENTZ, E., BORNERT, J. M., MESSADDEQ, N., WENDLING, O., MARK, M., DESVERGNE, B., WAHLI, W., CHAMBON, P. & METZGER, D. 2004. Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. *Proc Natl Acad Sci U S A,* 101**,** 4543-7.

INGVARTSEN, K. L. & ANDERSEN, J. B. 2000. Integration of Metabolism and Intake Regulation: A Review Focusing on Periparturient Animals. *Journal of Dairy Science,* 83**,** 1573-1597.
IRELAND, J. J., ROBERTS, R. M., PALMER, G. H., BAUMAN, D. E. & BAZER, F. W. 2008. A commentary on domestic animals as dual-purpose models that benefit agricultural and biomedical research. *J Anim Sci,* 86**,** 2797-805.
IRINTCHEV, A., ROLLENHAGEN, A., TRONCOSO, E., KISS, J. Z. & SCHACHNER, M. 2005. Structural and Functional Aberrations in the Cerebral Cortex of Tenascin-C Deficient Mice. *Cerebral Cortex,* 15**,** 950-962.
ISHIMOTO, Y., SATSU, H., TOTSUKA, M. & SHIMIZU, M. 2011. IEX-1 suppresses apoptotic damage in human intestinal epithelial Caco-2 cells induced by co-culturing with macrophage-like THP-1 cells. *Biosci Rep,* 31**,** 345-51.
IUCN. 1994. *1994 Categories & Criteria (version 2.3)* [Online]. Cambridge, UK: IUCN. [Accessed 15 July 2015].
IVERSEN, E. 1996. The domestication of African elephants. *Pachyderm,* 21**,** 65-68.
IWAKI, M., MATSUDA, M., MAEDA, N., FUNAHASHI, T., MATSUZAWA, Y., MAKISHIMA, M. & SHIMOMURA, I. 2003. Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes,* 52**,** 1655-63.
JAKOBSSON, A., WESTERBERG, R. & JACOBSSON, A. 2006. Fatty acid elongases in mammals: their regulation and roles in metabolism. *Prog Lipid Res,* 45**,** 237-49.
JENKINS, T. C. 1993. Lipid metabolism in the rumen. *J Dairy Sci,* 76**,** 3851-63.
JENKINS, T. C., KLEIN, C. M. & LEE, Y. J. New insights on the pathways of lipid biohydrogenation in the rumen with possible implications on animal performance. 24th Annual Southwest Nutrition and Management Conference, 2009. University of Arizona.
JHEON, A. H., SEIDEL, K., BIEHS, B. & KLEIN, O. D. 2013. From molecules to mastication: the development and evolution of teeth. *Wiley interdisciplinary reviews. Developmental biology,* 2**,** 165-183.
JIANG, Y., ZHAO, X., XIAO, Q., LIU, Q., DING, K., YU, F., ZHANG, R., ZHU, T. & GE, G. 2014. Snail and Slug mediate tamoxifen resistance in breast cancer cells through activation of EGFR-ERK independent of epithelial-mesenchymal transition. *J Mol Cell Biol.* United States.
JIMENEZ, M. A., AKERBLAD, P., SIGVARDSSON, M. & ROSEN, E. D. 2007. Critical role for Ebf1 and Ebf2 in the adipogenic transcriptional cascade. *Mol Cell Biol,* 27**,** 743-57.
JITRAPAKDEE, S., MAURICE, M. S., RAYMENT, I., CLELAND, W. W., WALLACE, J. C. & ATTWOOD, P. V. 2008. Structure, Mechanism and Regulation of Pyruvate Carboxylase. *The Biochemical journal,* 413**,** 369-387.
JO, J., GAVRILOVA, O., PACK, S., JOU, W., MULLEN, S., SUMNER, A. E., CUSHMAN, S. W. & PERIWAL, V. 2009. Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. *PLoS Comput Biol,* 5**,** e1000324.
JOHNSON, G. A., BURGHARDT, R. C., BAZER, F. W. & SPENCER, T. E. 2003. Osteopontin: Roles in Implantation and Placentation. *Biology of Reproduction,* 69**,** 1458-1471.
JOHNSON, M., BRACKELSBERG, P. O., ROTHSCHILD, M. F. & CHENG, P. 1995. *Meishan* [Online]. Oklahoma, USA: Oklahoma State University Board of Regents. Available: http://www.ansi.okstate.edu/breeds/swine/meishan/ [Accessed 20 October 2014].
JOHNSTON, C. S., DAY, C. S. & SWAN, P. D. 2002. Postprandial thermogenesis is increased 100% on a high-protein, low-fat diet versus a high-carbohydrate, low-fat diet in healthy, young women. *J Am Coll Nutr,* 21**,** 55-61.
JOHNSTONE, A. M., HORGAN, G. W., MURISON, S. D., BREMNER, D. M. & LOBLEY, G. E. 2008. Effects of a high-protein ketogenic diet on hunger,

appetite, and weight loss in obese men feeding ad libitum. *Am J Clin Nutr,* 87**,** 44-55.
JONES, D. K., LIU, F., VAIDYANATHAN, R., ECKHARDT, L. L., TRUDEAU, M. C. & ROBERTSON, G. A. 2014. hERG 1b is critical for human cardiac repolarization. *Proc Natl Acad Sci U S A,* 111**,** 18073-7.
JORDAN, C. D., BOHLING, S. D., CHARBONNEAU, N. L. & SAKAI, L. Y. 2010. Fibrillins in adult human ovary and polycystic ovary syndrome: is fibrillin-3 affected in PCOS? *J Histochem Cytochem,* 58**,** 903-15.
JOSHI, N. A. & FASS, J. N. 2011. Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33). 1.33 ed. San Francisco: GitHub.
JUNGERT, A., ROTH, H. J. & NEUHAUSER-BERTHOLD, M. 2012. Serum 25-hydroxyvitamin D3 and body composition in an elderly cohort from Germany: a cross-sectional study. *Nutr Metab (Lond),* 9**,** 42.
KASTURI, R. & JOSHI, V. C. 1982. Hormonal regulation of stearoyl coenzyme A desaturase activity and lipogenesis during adipose conversion of 3T3-L1 cells. *J Biol Chem,* 257**,** 12224-30.
KATOH, M. 2003. WNT2 and human gastrointestinal cancer (review). *Int J Mol Med,* 12**,** 811-6.
KEISLER, D. H., DANIEL, J. A. & MORRISON, C. D. 1999. The role of leptin in nutritional status and reproductive function. *J Reprod Fertil Suppl,* 54**,** 425-35.
KENNEDY, A., GETTYS, T. W., WATSON, P., WALLACE, P., GANAWAY, E., PAN, Q. & GARVEY, W. T. 1997. The metabolic significance of leptin in humans: gender-based differences in relationship to adiposity, insulin sensitivity, and energy expenditure. *J Clin Endocrinol Metab,* 82**,** 1293-300.
KERSHAW, E. E. & FLIER, J. S. 2004. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab,* 89**,** 2548-56.
KERSTEN, S. 2001. Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Reports,* 2**,** 282-286.
KHATIB, H., ZAITOUN, I., WIEBELHAUS-FINGER, J., CHANG, Y. M. & ROSA, G. J. 2007. The association of bovine PPARGC1A and OPN genes with milk composition in two independent Holstein cattle populations. *J Dairy Sci,* 90**,** 2966-70.
KIM, J. B. & SPIEGELMAN, B. M. 1996. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev,* 10**,** 1096-107.
KIM, Y. C. & NTAMBI, J. M. 1999. Regulation of stearoyl-CoA desaturase genes: role in cellular metabolism and preadipocyte differentiation. *Biochem Biophys Res Commun,* 266**,** 1-4.
KIM, Y. J., HWANG, S. H., CHO, H. H., SHIN, K. K., BAE, Y. C. & JUNG, J. S. 2012. MicroRNA 21 regulates the proliferation of human adipose tissue-derived mesenchymal stem cells and high-fat diet-induced obesity alters microRNA 21 expression in white adipose tissues. *J Cell Physiol,* 227**,** 183-93.
KING, P. 2004a. The birth of breeds. *In:* FERRETTI, E. (ed.) *Tradition cattle breeds and how to keep them.* Preston, UK: Farming Books and Videos Limited.
KING, P. 2004b. The origins and history of cattle. *In:* FERRETTI, E. (ed.) *Tradition cattle breeds and how to keep them.* Preston, UK: Farming Books and Videos Limited.
KINOSHITA, T. & ITOH, M. 2006. Longitudinal variance of fat mass deposition during pregnancy evaluated by ultrasonography: the ratio of visceral fat to subcutaneous fat in the abdomen. *Gynecol Obstet Invest,* 61**,** 115-8.
KIRKWOOD, R. N. & AHERNE, F. X. 1985. Energy intake, body composition and reproductive performance of the gilt. *J Anim Sci,* 60**,** 1518-29.
KIRKWOOD, R. N. & THACKER, P. A. 1992. Management of replacement breeding animals. *Vet Clin North Am Food Anim Pract,* 8**,** 575-87.

KLEIN, C. E. 2003. The Hypothalamic-Pituitary-Gonadal Axis. *In:* KUFE, D. W. P., R.E.WEICHSELBAUM, R.R. (ed.) *Holland-Frei Cancer Medicine.* 6th ed. Hamilton, Ontario: BC Decker.

KLOK, M. D., JAKOBSDOTTIR, S. & DRENT, M. L. 2007. The role of leptin and ghrelin in the regulation of food intake and body weight in humans: a review. *Obes Rev,* 8**,** 21-34.

KNITTLE, J. L., TIMMERS, K., GINSBERG-FELLNER, F., BROWN, R. E. & KATZ, D. P. 1979. The growth of adipose tissue in children and adolescents. Cross-sectional and longitudinal studies of adipose cell number and size. *J Clin Invest,* 63**,** 239-46.

KOMAR, C. M., BRAISSANT, O., WAHLI, W. & CURRY, T. E., JR. 2001. Expression and localization of PPARs in the rat ovary during follicular development and the periovulatory period. *Endocrinology,* 142**,** 4831-8.

KONG, J., CHEN, Y., ZHU, G., ZHAO, Q. & LI, Y. C. 2013. 1,25-Dihydroxyvitamin D3 upregulates leptin expression in mouse adipose tissue. *J Endocrinol,* 216**,** 265-71.

KONG, J. & LI, Y. C. 2006. Molecular mechanism of 1,25-dihydroxyvitamin D3 inhibition of adipogenesis in 3T3-L1 cells. *Am J Physiol Endocrinol Metab,* 290**,** E916-24.

KONG, X., BANKS, A., LIU, T., KAZAK, L., RAO, R. R., COHEN, P., WANG, X., YU, S., LO, J. C., TSENG, Y. H., CYPESS, A. M., XUE, R., KLEINER, S., KANG, S., SPIEGELMAN, B. M. & ROSEN, E. D. 2014. IRF4 is a key thermogenic transcriptional partner of PGC-1alpha. *Cell,* 158**,** 69-83.

KONNECKE, M., BOSCKE, R., WALDMANN, A., BRUCHHAGE, K. L., LINKE, R., PRIES, R. & WOLLENBERG, B. 2014. Immune imbalance in nasal polyps of Caucasian chronic rhinosinusitis patients is associated with a downregulation of E-selectin. *J Immunol Res,* 2014**,** 959854.

KOURI, V. P., OLKKONEN, J., AINOLA, M., LI, T. F., BJORKMAN, L., KONTTINEN, Y. T. & MANDELIN, J. 2014. Neutrophils produce interleukin-17B in rheumatoid synovial tissue. *Rheumatology (Oxford),* 53**,** 39-47.

KOUTAKIS, P., MISERLIS, D., MYERS, S. A., KIM, J. K., ZHU, Z., PAPOUTSI, E., SWANSON, S. A., HAYNATZKI, G., HA, D. M., CARPENTER, L. A., MCCOMB, R. D., JOHANNING, J. M., CASALE, G. P. & PIPINOS, II 2015. Abnormal accumulation of desmin in gastrocnemius myofibers of patients with peripheral artery disease: associations with altered myofiber morphology and density, mitochondrial dysfunction and impaired limb function. *J Histochem Cytochem,* 63**,** 256-69.

KOZAK, L. P. & ANUNCIADO-KOZA, R. 2008. UCP1: its involvement and utility in obesity. *Int J Obes (Lond),* 32 Suppl 7**,** S32-8.

KOZAK, L. P. & HARPER, M. E. 2000. Mitochondrial uncoupling proteins in energy expenditure. *Annu Rev Nutr,* 20**,** 339-63.

KOZERA, B. & RAPACZ, M. 2013. Reference genes in real-time PCR. *J Appl Genet,* 54**,** 391-406.

KRAEMER, F. B. & SHEN, W.-J. 2002. Hormone-sensitive lipase: control of intracellular tri-(di-)acylglycerol and cholesteryl ester hydrolysis. *Journal of Lipid Research,* 43**,** 1585-1594.

KRAMER, A., GREEN, J., POLLARD, J., JR. & TUGENDREICH, S. 2014. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics,* 30**,** 523-30.

KREMPLER, F., BREBAN, D., OBERKOFLER, H., ESTERBAUER, H., HELL, E., PAULWEBER, B. & PATSCH, W. 2000. Leptin, Peroxisome Proliferator-Activated Receptor-γ, and CCAAT/Enhancer Binding Protein-α mRNA Expression in Adipose Tissue of Humans and Their Relation to Cardiovascular Risk Factors. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 20**,** 443-449.

KRISTIANSSON, E., OSTERLUND, T., GUNNARSSON, L., ARNE, G., LARSSON, D. G. & NERMAN, O. 2013. A novel method for cross-species gene expression analysis. *BMC Bioinformatics,* 14**,** 70.

KUMAR, M. V. & SCARPACE, P. J. 1998. Differential effects of retinoic acid on uncoupling protein-1 and leptin gene expression. *J Endocrinol,* 157**,** 237-43.

KURT, F. & MAR, D. K. 1996. Neonate mortality in captive Asian elephants (Elephas maximus). *Zeitschrift für Säugetierkunde,* 61**,** 155-164

KUYZNIEREWICZ, I. & THOMSON, M. 2002. GTP-binding proteins G(salpha), G(ialpha), and Ran identified in mitochondria of human placenta. *Cell Biol Int,* 26**,** 99-108.

LAI, N., SIMS, J. K., JEON, N. L. & LEE, K. 2012. Adipocyte Induction of Preadipocyte Differentiation in a Gradient Chamber. *Tissue Engineering. Part C, Methods,* 18**,** 958-967.

LAIN, K. Y. & CATALANO, P. M. 2007. Metabolic changes in pregnancy. *Clin Obstet Gynecol,* 50**,** 938-48.

LALIOTIS, G. P., BIZELIS, I. & ROGDAKIS, E. 2010. Comparative Approach of the de novo Fatty Acid Synthesis (Lipogenesis) between Ruminant and Non Ruminant Mammalian Species: From Biochemical Level to the Main Regulatory Lipogenic Genes. *Curr Genomics,* 11**,** 168-83.

LAMMERS, B. P., HEINRICHS, A. J. & KENSINGER, R. S. 1999. The effects of accelerated growth rates and estrogen implants in prepubertal Holstein heifers on estimates of mammary development and subsequent reproduction and milk production. *J Dairy Sci,* 82**,** 1753-64.

LANDIS, M. D., CARSTENS, G. E., MCPHAIL, E. G., RANDEL, R. D., GREEN, K. K., SLAY, L. & SMITH, S. B. 2002. Ontogenic development of brown adipose tissue in Angus and Brahman fetal calves. *J Anim Sci,* 80**,** 591-601.

LANDSTROM, A. P., PARVATIYAR, M. S., PINTO, J. R., MARQUARDT, M. L., BOS, J. M., TESTER, D. J., OMMEN, S. R., POTTER, J. D. & ACKERMAN, M. J. 2008. Molecular and functional characterization of novel hypertrophic cardiomyopathy susceptibility mutations in TNNC1-encoded troponin C. *J Mol Cell Cardiol,* 45**,** 281-8.

LANG, F., HUANG, D. Y. & VALLON, V. 2010. SGK, renal function and hypertension. *J Nephrol,* 23 Suppl 16**,** S124-9.

LANZAS, C., AYSCUE, P., IVANEK, R. & GROHN, Y. T. 2010. Model or meal? Farm animal populations as models for infectious diseases of humans. *Nat Rev Micro,* 8**,** 139-148.

LARSON, E. 2007. Community factors in the development of antibiotic resistance. *Annu Rev Public Health,* 28**,** 435-47.

LARSON, G., DOBNEY, K., ALBARELLA, U., FANG, M., MATISOO-SMITH, E., ROBINS, J., LOWDEN, S., FINLAYSON, H., BRAND, T., WILLERSLEV, E., ROWLEY-CONWY, P., ANDERSSON, L. & COOPER, A. 2005. Worldwide Phylogeography of Wild Boar Reveals Multiple Centers of Pig Domestication. *Science,* 307**,** 1618-1621.

LARSON, G., LIU, R., ZHAO, X., YUAN, J., FULLER, D., BARTON, L., DOBNEY, K., FAN, Q., GU, Z., LIU, X.-H., LUO, Y., LV, P., ANDERSSON, L. & LI, N. 2010. Patterns of East Asian pig domestication, migration, and turnover revealed by modern and ancient DNA. *Proceedings of the National Academy of Sciences,* 107**,** 7686-7691.

LAUER, M. E., LOFTIS, J., DE LA MOTTE, C. & HASCALL, V. C. 2015. Analysis of the heavy-chain modification and TSG-6 activity in pathological hyaluronan matrices. *Methods Mol Biol,* 1229**,** 543-8.

LAWS, R. M. 1967. Occurrence of placental scars in the uterus of the African elephant (Loxodonta africana). *Journal of Reproduction and Fertility,* 14**,** 445-449.

LAY, S. L., LEFRÈRE, I., TRAUTWEIN, C., DUGAIL, I. & KRIEF, S. 2002. Insulin and Sterol-regulatory Element-binding Protein-1c (SREBP-1C) Regulation of

Gene Expression in 3T3-L1 Adipocytes: IDENTIFICATION OF CCAAT/ENHANCER-BINDING PROTEIN β AS AN SREBP-1C TARGET. *Journal of Biological Chemistry,* 277**,** 35625-35634.

LEADER-WILLIAMS, N. & ALBON, S. D. 1988. Allocation of resources for conservation. *Nature,* 336**,** 533-535.

LEBLANC, S. 2010. Assessing the association of the level of milk production with reproductive performance in dairy cattle. *J Reprod Dev,* 56 Suppl**,** S1-7.

LEBRET, B. 2008. Effects of feeding and rearing systems on growth, carcass composition and meat quality in pigs. *Animal,* 2**,** 1548-58.

LEE, G. J., RITCHIE, M., THOMSON, M., MACDONALD, A. A., BLASCO, A., SANTACREU, M. A., ARGENTE, M. J. & HALEY, C. S. 1995. Uterine capacity and prenatal survival in Meishan and Large White pigs. *Animal Science,* 60**,** 471-479.

LEE, H. Y., AHN, J. B., RHA, S. Y., CHUNG, H. C., PARK, K. H., KIM, T. S., KIM, N. K. & SHIN, S. J. 2014. High KLF4 level in normal tissue predicts poor survival in colorectal cancer patients. *World J Surg Oncol,* 12**,** 232.

LEFEBVRE, A.-M., LAVILLE, M., VEGA, N., RIOU, J. P., GAAL, L. V., AUWERX, J. & VIDAL, H. 1998. Depot-Specific Differences in Adipose Tissue Gene Expression in Lean and Obese Subjects. *Diabetes,* 47**,** 98-103.

LEFTEROVA, M. I., ZHANG, Y., STEGER, D. J., SCHUPP, M., SCHUG, J., CRISTANCHO, A., FENG, D., ZHUO, D., STOECKERT, C. J., JR., LIU, X. S. & LAZAR, M. A. 2008. PPARgamma and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. *Genes Dev,* 22**,** 2941-52.

LEHTONEN, H. J., SIPPONEN, T., TOJKANDER, S., KARIKOSKI, R., JARVINEN, H., LAING, N. G., LAPPALAINEN, P., AALTONEN, L. A. & TUUPANEN, S. 2012. Segregation of a missense variant in enteric smooth muscle actin gamma-2 with autosomal dominant familial visceral myopathy. *Gastroenterology,* 143**,** 1482-1491.e3.

LEINNINGER, G. M., JO, Y. H., LESHAN, R. L., LOUIS, G. W., YANG, H., BARRERA, J. G., WILSON, H., OPLAND, D. M., FAOUZI, M. A., GONG, Y., JONES, J. C., RHODES, C. J., CHUA, S., JR., DIANO, S., HORVATH, T. L., SEELEY, R. J., BECKER, J. B., MUNZBERG, H. & MYERS, M. G., JR. 2009. Leptin acts via leptin receptor-expressing lateral hypothalamic neurons to modulate the mesolimbic dopamine system and suppress feeding. *Cell Metab,* 10**,** 89-98.

LEMIEUX, S., PRUD'HOMME, D., BOUCHARD, C., TREMBLAY, A. & DESPRES, J. P. 1993. Sex differences in the relation of visceral adipose tissue accumulation to total body fatness. *Am J Clin Nutr,* 58**,** 463-7.

LEONARD, A. E., BOBIK, E. G., DORADO, J., KROEGER, P. E., CHUANG, L. T., THURMOND, J. M., PARKER-BARNES, J. M., DAS, T., HUANG, Y. S. & MUKERJI, P. 2000. Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. *Biochemical Journal,* 350**,** 765-770.

LEONARD, S., KHATIB, H., SCHUTZKUS, V., CHANG, Y. M. & MALTECCA, C. 2005. Effects of the osteopontin gene variants on milk production traits in dairy cattle. *J Dairy Sci,* 88**,** 4083-6.

LEROY, J. L., VANHOLDER, T., MATEUSEN, B., CHRISTOPHE, A., OPSOMER, G., DE KRUIF, A., GENICOT, G. & VAN SOOM, A. 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction,* 130**,** 485-95.

LEVINE, M. N., BRAMWELL, V., PRITCHARD, K., PERRAULT, D., FINDLAY, B., ABU-ZAHRA, H., WARR, D., ARNOLD, A. & SKILLINGS, J. 1993. The Canadian experience with intensive fluorouracil, epirubicin and

cyclophosphamide in patients with early stage breast cancer. *Drugs,* 45 Suppl 2**,** 51-9; discussion 58-9.
LEWIS, A. M., WATERHOUSE, C. & JACOBS, L. S. 1980. Whole-blood and plasma amino acid analysis: gas-liquid and cation-exchange chromatography compared. *Clin Chem,* 26**,** 271-6.
LI, J. & CHENG, J.-X. 2014. Direct visualization of de novo lipogenesis in single living cells. *Sci. Rep.,* 4.
LI, J., TAKAISHI, K., COOK, W., MCCORKLE, S. K. & UNGER, R. H. 2003. Insig-1 "brakes" lipogenesis in adipocytes and inhibits differentiation of preadipocytes. *Proceedings of the National Academy of Sciences,* 100**,** 9476-9481.
LI, J., YU, X., PAN, W. & UNGER, R. H. 2002. Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity. *American Journal of Physiology - Endocrinology and Metabolism,* 282**,** E1334-E1341.
LI, L., XIE, X., QIN, J., JEHA, G. S., SAHA, P. K., YAN, J., HAUETER, C. M., CHAN, L., TSAI, S. Y. & TSAI, M. J. 2009. The nuclear orphan receptor COUP-TFII plays an essential role in adipogenesis, glucose homeostasis, and energy metabolism. *Cell Metab,* 9**,** 77-87.
LI, X., THOMASON, P. A., WITHERS, D. J. & SCOTT, J. 2010. Bio-informatics analysis of a gene co-expression module in adipose tissue containing the diet-responsive gene Nnat. *BMC Syst Biol.* England.
LI, X. J., YANG, H., LI, G. X., ZHANG, G. H., CHENG, J., GUAN, H. & YANG, G. S. 2012a. Transcriptome profile analysis of porcine adipose tissue by high-throughput sequencing. *Anim Genet,* 43**,** 144-52.
LI, Y., MOUCHE, S., SAJIC, T., VEYRAT-DUREBEX, C., SUPALE, R., PIERROZ, D., FERRARI, S., NEGRO, F., HASLER, U., FERAILLE, E., MOLL, S., MEDA, P., DEFFERT, C., MONTET, X., KRAUSE, K. H. & SZANTO, I. 2012b. Deficiency in the NADPH oxidase 4 predisposes towards diet-induced obesity. *Int J Obes (Lond),* 36**,** 1503-13.
LIM, R. C., PRICE, J. T. & WILCE, J. A. 2014. Context-dependent role of Grb7 in HER2+ve and triple-negative breast cancer cell lines. *Breast Cancer Res Treat,* 143**,** 593-603.
LIN, Y. S., CHENG, T. H., CHANG, C. P., CHEN, H. M. & CHERN, Y. 2013. Enhancement of brain-type creatine kinase activity ameliorates neuronal deficits in Huntington's disease. *Biochim Biophys Acta,* 1832**,** 742-53.
LINDEQUE, M. & JAARSVELD, A. S. V. 1993. Post-natal growth of elephants Loxodonta africana in Etosha National Park, Namibia. *Journal of Zoology,* 229**,** 319-330.
LIU, L., LI, Y., LI, S., HU, N., HE, Y., PONG, R., LIN, D., LU, L. & LAW, M. 2012a. Comparison of next-generation sequencing systems. *J Biomed Biotechnol,* 2012**,** 251364.
LIU, Y., LI, M., GUAN, J. & LI, X. 2012b. Breed, gender and anatomic location-specific expression patterns of the porcine insig-1 and insig-2 genes. *Scientific Research and Essays,* 7**,** 1573-1576.
LOCK, A. L. & BAUMAN, D. E. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. *Lipids,* 39**,** 1197-1206.
LOFTUS, R. T., ERTUGRUL, O., HARBA, A. H., EL-BARODY, M. A., MACHUGH, D. E., PARK, S. D. & BRADLEY, D. G. 1999. A microsatellite survey of cattle from a centre of origin: the Near East. *Mol Ecol.* England.
LONCAR, D., AFZELIUS, B. A. & CANNON, B. 1988a. Epididymal white adipose tissue after cold stress in rats. I. Nonmitochondrial changes. *J Ultrastruct Mol Struct Res,* 101**,** 109-22.
LONCAR, D., AFZELIUS, B. A. & CANNON, B. 1988b. Epididymal white adipose tissue after cold stress in rats. II. Mitochondrial changes. *J Ultrastruct Mol Struct Res,* 101**,** 199-209.

LOURENCO, M., RAMOS-MORALES, E. & WALLACE, R. J. 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. *Animal,* 4**,** 1008-23.

LU, D., ZHANG, L., BAO, D., LU, Y., ZHANG, X., LIU, N., GE, W., GAO, X. & LI, H. 2014. Calponin1 inhibits dilated cardiomyopathy development in mice through the epsilonPKC pathway. *Int J Cardiol,* 173**,** 146-53.

LUCY, M. C. 2001. Reproductive Loss in High-Producing Dairy Cattle: Where Will It End? *Journal of Dairy Science,* 84**,** 1277-1293.

LUO, A., YU, X., LI, G., MA, G., CHEN, H., DING, F., LI, Y. & LIU, Z. 2014. Differentiation-associated genes regulated by c-Jun and decreased in the progression of esophageal squamous cell carcinoma. *PLoS One,* 9**,** e96610.

LUTTINGER, D., KING, R. A., SHEPPARD, D., STRUPP, J., NEMEROFF, C. B. & PRANGE, A. J., JR. 1982. The effect of neurotensin on food consumption in the rat. *Eur J Pharmacol,* 81**,** 499-503.

LYNCH, C. J., FOX, H., HAZEN, S. A., STANLEY, B. A., DODGSON, S. & LANOUE, K. F. 1995. Role of hepatic carbonic anhydrase in de novo lipogenesis. *Biochem J,* 310 (Pt 1)**,** 197-202.

LYNCH, C. J., HAZEN, S. A., HORETSKY, R. L., CARTER, N. D. & DODGSON, S. J. 1993. Differentiation-dependent expression of carbonic anhydrase II and III in 3T3 adipocytes. *Am J Physiol,* 265**,** C234-43.

MACDONALD, K. A., VERKERK, G. A., THORROLD, B. S., PRYCE, J. E., PENNO, J. W., MCNAUGHTON, L. R., BURTON, L. J., LANCASTER, J. A., WILLIAMSON, J. H. & HOLMES, C. W. 2008. A comparison of three strains of holstein-friesian grazed on pasture and managed under different feed allowances. *J Dairy Sci,* 91**,** 1693-707.

MACDOUGAL, M. G. 2012. *Adipose tissue's potential role as a reproductive or lactation endocrine gland.* Master's of Science, University of Tennessee.

MACHINAL, F., DIEUDONNE, M. N., LENEVEU, M. C., PECQUERY, R. & GIUDICELLI, Y. 1999. In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: evidence for a regional specific regulation by sex steroid hormones. *Endocrinology,* 140**,** 1567-74.

MACIEL, M. N., ZIEBA, D. A., AMSTALDEN, M., KEISLER, D. H., NEVES, J. P. & WILLIAMS, G. L. 2004. Chronic administration of recombinant ovine leptin in growing beef heifers: effects on secretion of LH, metabolic hormones, and timing of puberty. *J Anim Sci,* 82**,** 2930-6.

MADEIRA, M. S., PIRES, V. M., ALFAIA, C. M., COSTA, A. S., LUXTON, R., DORAN, O., BESSA, R. J. & PRATES, J. A. 2013. Differential effects of reduced protein diets on fatty acid composition and gene expression in muscle and subcutaneous adipose tissue of Alentejana purebred and Large White x Landrace x Pietrain crossbred pigs. *Br J Nutr,* 110**,** 216-29.

MADEIRA, M. S., PIRES, V. M., ALFAIA, C. M., LUXTON, R., DORAN, O., BESSA, R. J. & PRATES, J. A. 2014. Combined effects of dietary arginine, leucine and protein levels on fatty acid composition and gene expression in the muscle and subcutaneous adipose tissue of crossbred pigs. *Br J Nutr,* 111**,** 1521-35.

MAES, D. G. D., JANSSENS, G. P. J., DELPUTTE, P., LAMMERTYN, A. & DE KRUIF, A. 2004. Back fat measurements in sows from three commercial pig herds: relationship with reproductive efficiency and correlation with visual body condition scores. *Livestock Production Science,* 91**,** 57-67.

MAGYAR, I., LEHOCZKY, D. & MARTON, I. 1966. Treatment of insulin resistant diabetes with porcine insulin. *Orv Hetil,* 107**,** 829-32.

MAHAJAN, A. & STAHL, C. H. 2009. Dihydroxy-cholecalciferol stimulates adipocytic differentiation of porcine mesenchymal stem cells. *J Nutr Biochem,* 20**,** 512-20.

MALAU-ADULI, A. E. O., SIEBERT, B. D., BOTTEMA, C. D. K. & PITCHFORD, W. S. 1997. A comparison of the fatty acid composition of triacylglycerols in

adipose tissue from Limousin and Jersey cattle. *Australian Journal of Agricultural Research,* 48**,** 715-722.

MANNERS, M. J. & MCCREA, M. R. 1963. Changes in the chemical composition of of sow-reared piglets during the 1st month of life. *Br J Nutr,* 17**,** 495-513.

MANOLESCU, D. C., EL-KARES, R., LAKHAL-CHAIEB, L., MONTPETIT, A., BHAT, P. V. & GOODYER, P. 2010. Newborn Serum Retinoic Acid Level Is Associated With Variants of Genes in the Retinol Metabolism Pathway. *Pediatr Res,* 67**,** 598-602.

MANTENA, S. R., KANNAN, A., CHEON, Y. P., LI, Q., JOHNSON, P. F., BAGCHI, I. C. & BAGCHI, M. K. 2006. C/EBPbeta is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma. *Proc Natl Acad Sci U S A,* 103**,** 1870-5.

MAR, K. U., LAHDENPERÄ, M. & LUMMAA, V. 2012. Causes and Correlates of Calf Mortality in Captive Asian Elephants (*Elephas maximus*). *PLoS ONE,* 7**,** e32335.

MARCELO, K. L., GOLDIE, L. C. & HIRSCHI, K. K. 2013. Regulation of Endothelial Cell Differentiation and Specification. *Circulation Research,* 112**,** 1272-1287.

MAREI, W. F., WATHES, D. C. & FOULADI-NASHTA, A. A. 2009. The effect of linolenic Acid on bovine oocyte maturation and development. *Biol Reprod,* 81**,** 1064-72.

MARKLJUNG, E., JIANG, L., JAFFE, J. D., MIKKELSEN, T. S., WALLERMAN, O., LARHAMMAR, M., ZHANG, X., WANG, L., SAENZ-VASH, V., GNIRKE, A., LINDROTH, A. M., BARRES, R., YAN, J., STROMBERG, S., DE, S., PONTEN, F., LANDER, E. S., CARR, S. A., ZIERATH, J. R., KULLANDER, K., WADELIUS, C., LINDBLAD-TOH, K., ANDERSSON, G., HJALM, G. & ANDERSSON, L. 2009. ZBED6, a novel transcription factor derived from a domesticated DNA transposon regulates IGF2 expression and muscle growth. *PLoS Biol,* 7**,** e1000256.

MARTIN, G. S., LUNT, D. K., BRITAIN, K. G. & SMITH, S. B. 1999. Postnatal development of stearoyl coenzyme A desaturase gene expression and adiposity in bovine subcutaneous adipose tissue. *J Anim Sci.,* 77**,** 630-636.

MARTIN, J. C., BOUGNOUX, P., FIGNON, A., THERET, V., ANTOINE, J. M., LAMISSE, F. & COUET, C. 1993. Dependence of human milk essential fatty acids on adipose stores during lactation. *The American Journal of Clinical Nutrition,* 58**,** 653-659.

MARTIN, J. C., NIYONGABO, T., MOREAU, L., ANTOINE, J. M., LANSON, M., BERGER, C., LAMISSE, F., BOUGNOUX, P. & COUET, C. 1991. Essential fatty acid composition of human colostrum triglycerides: its relationship with adipose tissue composition. *The American Journal of Clinical Nutrition,* 54**,** 829-835.

MARTIN, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data Analysis*.

MATSUZAKA, T. & SHIMANO, H. 2009. Elovl6: a new player in fatty acid metabolism and insulin sensitivity. *J Mol Med (Berl),* 87**,** 379-84.

MATSUZAKA, T., SHIMANO, H., YAHAGI, N., KATO, T., ATSUMI, A., YAMAMOTO, T., INOUE, N., ISHIKAWA, M., OKADA, S., ISHIGAKI, N., IWASAKI, H., IWASAKI, Y., KARASAWA, T., KUMADAKI, S., MATSUI, T., SEKIYA, M., OHASHI, K., HASTY, A. H., NAKAGAWA, Y., TAKAHASHI, A., SUZUKI, H., YATOH, S., SONE, H., TOYOSHIMA, H., OSUGA, J.-I. & YAMADA, N. 2007. Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. *Nat Med,* 13**,** 1193-1202.

MATSUZAKA, T., SHIMANO, H., YAHAGI, N., YOSHIKAWA, T., AMEMIYA-KUDO, M., HASTY, A. H., OKAZAKI, H., TAMURA, Y., IIZUKA, Y., OHASHI, K., OSUGA, J., TAKAHASHI, A., YATO, S., SONE, H., ISHIBASHI, S. &

YAMADA, N. 2002. Cloning and characterization of a mammalian fatty acyl-CoA elongase as a lipogenic enzyme regulated by SREBPs. *J Lipid Res,* 43**,** 911-20.
MATTOS, R., STAPLES, C. R. & THATCHER, W. W. 2000. Effects of dietary fatty acids on reproduction in ruminants. *Rev Reprod,* 5**,** 38-45.
MAUMUS, M., SENGENES, C., DECAUNES, P., ZAKAROFF-GIRARD, A., BOURLIER, V., LAFONTAN, M., GALITZKY, J. & BOULOUMIE, A. 2008. Evidence of in situ proliferation of adult adipose tissue-derived progenitor cells: influence of fat mass microenvironment and growth. *J Clin Endocrinol Metab,* 93**,** 4098-106.
MCCARTY, M. F. 2001. Modulation of adipocyte lipoprotein lipase expression as a strategy for preventing or treating visceral obesity. *Med Hypotheses,* 57**,** 192-200.
MECKLING, K. A., GAUTHIER, M., GRUBB, R. & SANFORD, J. 2002. Effects of a hypocaloric, low-carbohydrate diet on weight loss, blood lipids, blood pressure, glucose tolerance, and body composition in free-living overweight women. *Can J Physiol Pharmacol,* 80**,** 1095-105.
MEETHAL, S. V., LIU, T., CHAN, H. W., GINSBURG, E., WILSON, A. C., GRAY, D. N., BOWEN, R. L., VONDERHAAR, B. K. & ATWOOD, C. S. 2009. Identification of a regulatory loop for the synthesis of neurosteroids: a steroidogenic acute regulatory protein-dependent mechanism involving hypothalamic-pituitary-gonadal axis receptors. *Journal of Neurochemistry,* 110**,** 1014-1027.
MEGENS, H. J., CROOIJMANS, R. P., SAN CRISTOBAL, M., HUI, X., LI, N. & GROENEN, M. A. 2008. Biodiversity of pig breeds from China and Europe estimated from pooled DNA samples: differences in microsatellite variation between two areas of domestication. *Genet Sel Evol,* 40**,** 103-28.
MELINA, R. 2010. *Why do researchers use mice?* [Online]. Ogden, UT: Live Science. [Accessed 15 September 2015 2015].
MENENDEZ, C., LAGE, M., PEINO, R., BALDELLI, R., CONCHEIRO, P., DIEGUEZ, C. & CASANUEVA, F. F. 2001. Retinoic acid and vitamin D(3) powerfully inhibit in vitro leptin secretion by human adipose tissue. *J Endocrinol,* 170**,** 425-31.
MENENDEZ, J. A. & LUPU, R. 2007. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer,* 7**,** 763-777.
MERCADER, J., RIBOT, J., MURANO, I., FELIPE, F., CINTI, S., BONET, M. L. & PALOU, A. 2006. Remodeling of white adipose tissue after retinoic acid administration in mice. *Endocrinology,* 147**,** 5325-32.
METZGER, D., IMAI, T., JIANG, M., TAKUKAWA, R., DESVERGNE, B., WAHLI, W. & CHAMBON, P. 2005. Functional role of RXRs and PPARgamma in mature adipocytes. *Prostaglandins Leukot Essent Fatty Acids,* 73**,** 51-8.
MIGITA, T., OKABE, S., IKEDA, K., IGARASHI, S., SUGAWARA, S., TOMIDA, A., SOGA, T., TAGUCHI, R. & SEIMIYA, H. 2014. Inhibition of ATP citrate lyase induces triglyceride accumulation with altered fatty acid composition in cancer cells. *Int J Cancer,* 135**,** 37-47.
MIKKELSEN, P. B., TOUBRO, S. & ASTRUP, A. 2000. Effect of fat-reduced diets on 24-h energy expenditure: comparisons between animal protein, vegetable protein, and carbohydrate. *Am J Clin Nutr,* 72**,** 1135-41.
MILLAR, R. P., LU, Z.-L., PAWSON, A. J., FLANAGAN, C. A., MORGAN, K. & MAUDSLEY, S. R. 2004. Gonadotropin-Releasing Hormone Receptors. *Endocrine Reviews,* 25**,** 235-275.
MINGE, C. E., ROBKER, R. L. & NORMAN, R. J. 2008. PPAR Gamma: Coordinating Metabolic and Immune Contributions to Female Fertility. *PPAR Res,* 2008**,** 243791.

MINGE, C. E., RYAN, N. K., HOEK, K. H. V. D., ROBKER, R. L. & NORMAN, R. J. 2006. Troglitazone Regulates Peroxisome Proliferator-Activated Receptors and Inducible Nitric Oxide Synthase in Murine Ovarian Macrophages. *Biology of Reproduction,* 74**,** 153-160.

MITCHELL, A. D., SCHOLZ, A. M. & MERSMANN, H. J. 2001. Growth and body composition. *In:* POND, W. G. & MERSMANN, H. J. (eds.) *The biology of the domestic pig.* New York, USA: Cornell University Press.

MITTERBERGER, M. C., KIM, G., ROSTEK, U., LEVINE, R. L. & ZWERSCHKE, W. 2012. Carbonic anhydrase III regulates peroxisome proliferator-activated receptor-γ2. *Experimental Cell Research,* 318**,** 877-886.

MIYAZAKI, M. & NTAMBI, J. M. 2003. Role of stearoyl-coenzyme A desaturase in lipid metabolism. *Prostaglandins Leukot Essent Fatty Acids,* 68**,** 113-21.

MOHAMED-ALI, V., PINKNEY, J. H. & COPPACK, S. W. 1998. Adipose tissue as an endocrine and paracrine organ. *Int J Obes Relat Metab Disord,* 22**,** 1145-58.

MOLLER, D. E. 2000. Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. *Trends Endocrinol Metab,* 11**,** 212-7.

MOON, Y.-A., HAMMER, R. E. & HORTON, J. D. 2009. Deletion of ELOVL5 leads to fatty liver through activation of SREBP-1c in mice. *Journal of Lipid Research,* 50**,** 412-423.

MOORE, J. D. 2001. The Ran-GTPase and cell-cycle control. *Bioessays,* 23**,** 77-85.

MOREIRA, F., CORCINI, C. D., MONDADORI, R. G., GEVEHR-FERNANDES, C., MENDES, F. F., ARAUJO, E. G. & LUCIA, T., JR. 2013. Leptin and mitogen-activated protein kinase (MAPK) in oocytes of sows and gilts. *Anim Reprod Sci,* 139**,** 89-94.

MORENO-NAVARRETE, J. M., ORTEGA, F., SERRANO, M., RODRIGUEZ-HERMOSA, J. I., RICART, W., MINGRONE, G. & FERNANDEZ-REAL, J. M. 2014. CIDEC/FSP27 and PLIN1 gene expression run in parallel to mitochondrial genes in human adipose tissue, both increasing after weight loss. *Int J Obes,* 38**,** 865-872.

MORFELD, K. A. & BROWN, J. L. 2014. Ovarian acyclicity in zoo African elephants (Loxodonta africana) is associated with high body condition scores and elevated serum insulin and leptin. *Reproduction, Fertility and Development*.

MORFELD, K. A., LEHNHARDT, J., ALLIGOOD, C., BOLLING, J. & BROWN, J. L. 2014. Development of a body condition scoring index for female African elephants validated by ultrasound measurements of subcutaneous fat. *PLoS ONE,* 9**,** e93802.

MOSTYN, A., ATTIG, L., LARCHER, T., DOU, S., CHAVATTE-PALMER, P., BOUKTHIR, M., GERTLER, A., DJIANE, J., E SYMONDS, M. & ABDENNEBI-NAJAR, L. 2014. UCP1 is present in porcine adipose tissue and is responsive to postnatal leptin. *Journal of Endocrinology,* 223**,** M31-M38.

MURUGANANDAN, S., PARLEE, S. D., ROURKE, J. L., ERNST, M. C., GORALSKI, K. B. & SINAL, C. J. 2011. Chemerin, a novel peroxisome proliferator-activated receptor gamma (PPARgamma) target gene that promotes mesenchymal stem cell adipogenesis. *J Biol Chem,* 286**,** 23982-95.

MÅNSSON, H. L. 2008. Fatty acids in bovine milk fat. *Food & Nutrition Research,* 52**,** 10.3402/fnr.v52i0.1821.

NAGATANI, S., GUTHIKONDA, P., THOMPSON, R. C., TSUKAMURA, H., MAEDA, K. I. & FOSTER, D. L. 1998. Evidence for GnRH regulation by leptin: leptin administration prevents reduced pulsatile LH secretion during fasting. *Neuroendocrinology,* 67**,** 370-6.

NAGY, T. R., GORAN, M. I., WEINSIER, R. L., TOTH, M. J., SCHUTZ, Y. & POEHLMAN, E. T. 1996. Determinants of basal fat oxidation in healthy Caucasians. *J Appl Physiol (1985),* 80**,** 1743-8.

NAIR, R. R., KHANNA, A. & SINGH, K. 2015. Association of increased S100A8 serum protein with early pregnancy loss. *Am J Reprod Immunol,* 73**,** 91-4.

NARVAEZ, C. J., MATTHEWS, D., BROUN, E., CHAN, M. & WELSH, J. 2009. Lean phenotype and resistance to diet-induced obesity in vitamin D receptor knockout mice correlates with induction of uncoupling protein-1 in white adipose tissue. *Endocrinology,* 150**,** 651-61.

NARVAEZ, C. J., SIMMONS, K. M., BRUNTON, J., SALINERO, A., CHITTUR, S. V. & WELSH, J. E. 2013. Induction of STEAP4 correlates with 1,25-dihydroxyvitamin D3 stimulation of adipogenesis in mesenchymal progenitor cells derived from human adipose tissue. *Journal of Cellular Physiology,* 228**,** 2024-2036.

NELSON, D. L. & COX, M. M. 2004a. Carbohydrates and glycobiology. *Lehninger Principles of Biochemistry.* 4th ed. New York: W. H. Freeman.

NELSON, D. L. & COX, M. M. 2004b. Storage lipids. *Lehninger Principles of Biochemistry.* 4th ed. New York: W. H. Freeman.

NELSON, L. R. & BULUN, S. E. 2001. Estrogen production and action. *J Am Acad Dermatol,* 45**,** S116-24.

NEWELL-FUGATE, A. E., TAIBL, J. N., CLARK, S. G., ALLOOSH, M., STUREK, M. & KRISHER, R. L. 2014. Effects of diet-induced obesity on metabolic parameters and reproductive function in female Ossabaw minipigs. *Comp Med,* 64**,** 44-9.

NFU 2013. Great British beef week. Warwickshire, UK: National Farmers' Union.

NFU 2014. Annual Review 2013. http://www.nfuonline.com/490-14tl-annual-review-2013-low-res/ ed. Warwickshire, UK: National Farmers' Union.

NG, S. K., NEO, S. Y., YAP, Y. W., KARUTURI, R. K., LOH, E. S., LIAU, K. H. & REN, E. C. 2009. Ablation of phosphoinositide-3-kinase class II alpha suppresses hepatoma cell proliferation. *Biochem Biophys Res Commun,* 387**,** 310-5.

NGAPO, T. M., FORTIN, J., AALHUS, J. L. & MARTIN, J. F. 2010. Consumer choices of pork chops: Results from two Canadian sites. *Food Research International,* 43**,** 1559-1565.

NGAPO, T. M., MARTIN, J. F. & DRANSFIELD, E. 2004. Consumer choices of pork chops: results from three panels in France. *Food Quality and Preference,* 15**,** 349-359.

NGAPO, T. M., MARTIN, J. F. & DRANSFIELD, E. 2007. International preferences for pork appearance: I. Consumer choices. *Food Quality and Preference,* 18**,** 26-36.

NGUYEN, T. T., MIJARES, A. H., JOHNSON, C. M. & JENSEN, M. D. 1996. Postprandial leg and splanchnic fatty acid metabolism in nonobese men and women. *American Journal of Physiology - Endocrinology and Metabolism,* 271**,** E965-E972.

NIELSEN, R., PEDERSEN, T. A., HAGENBEEK, D., MOULOS, P., SIERSBAEK, R., MEGENS, E., DENISSOV, S., BORGESEN, M., FRANCOIJS, K. J., MANDRUP, S. & STUNNENBERG, H. G. 2008. Genome-wide profiling of PPARgamma:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. *Genes Dev,* 22**,** 2953-67.

NILSSON, E. M., FAINBERG, H. P., CHOONG, S. S., GILES, T. C., SELLS, J., MAY, S., STANSFIELD, F. J., ALLEN, W. R., EMES, R. D., MOSTYN, A., MONGAN, N. P. & YON, L. 2014. Molecular characterization of adipose tissue in the African elephant (Loxodonta africana). *PLoS One,* 9**,** e91717.

NIMITPHONG, H., HOLICK, M. F., FRIED, S. K. & LEE, M. J. 2012. 25-hydroxyvitamin D(3) and 1,25-dihydroxyvitamin D(3) promote the differentiation of human subcutaneous preadipocytes. *PLoS One,* 7**,** e52171.

NOGALSKA, A. & SWIERCZYNSKI, J. 2001. The age-related differences in obese and fatty acid synthase gene expression in white adipose tissue of rat. *Biochim Biophys Acta,* 1533**,** 73-80.

NOGALSKA, A. & SWIERCZYNSKI, J. 2004. Potential role of high serum leptin concentration in age-related decrease of fatty acid synthase gene expression in rat white adipose tissue. *Exp Gerontol,* 39**,** 147-50.

NOMURA, Y., MOMOI, N., HIRONO, K., HATA, Y., TAKASAKI, A., NISHIDA, N. & ICHIDA, F. 2015. A novel MYH7 gene mutation in a fetus with left ventricular noncompaction. *Can J Cardiol,* 31**,** 103.e1-3.

NTAMBI, J. M., BUHROW, S. A., KAESTNER, K. H., CHRISTY, R. J., SIBLEY, E., KELLY, T. J., JR. & LANE, M. D. 1988. Differentiation-induced gene expression in 3T3-L1 preadipocytes. Characterization of a differentially expressed gene encoding stearoyl-CoA desaturase. *J Biol Chem,* 263**,** 17291-300.

NTAMBI, J. M., MIYAZAKI, M., STOEHR, J. P., LAN, H., KENDZIORSKI, C. M., YANDELL, B. S., SONG, Y., COHEN, P., FRIEDMAN, J. M. & ATTIE, A. D. 2002. Loss of stearoyl–CoA desaturase-1 function protects mice against adiposity. *Proceedings of the National Academy of Sciences,* 99**,** 11482-11486.

OETTINGER, T., JØRGENSEN, M., LADEFOGED, A., HASLØV, K. & ANDERSEN, P. 1999. Development of the Mycobacterium bovis BCG vaccine: review of the historical and biochemical evidence for a genealogical tree. *Tubercle and Lung Disease,* 79**,** 243-250.

OFFENBERG, H. H., SCHALK, J. A., MEUWISSEN, R. L., VAN AALDEREN, M., KESTER, H. A., DIETRICH, A. J. & HEYTING, C. 1998. SCP2: a major protein component of the axial elements of synaptonemal complexes of the rat. *Nucleic Acids Res,* 26**,** 2572-9.

OLIVEIRA-FERRER, L., ROSSLER, K., HAUSTEIN, V., SCHRODER, C., WICKLEIN, D., MALTSEVA, D., KHAUSTOVA, N., SAMATOV, T., TONEVITSKY, A., MAHNER, S., JANICKE, F., SCHUMACHER, U. & MILDE-LANGOSCH, K. 2014. c-FOS suppresses ovarian cancer progression by changing adhesion. *Br J Cancer,* 110**,** 753-63.

OLSON, D. & WIESE, R. J. 2000. State of the North American African elephant population and projections for the future. *Zoo Biology,* 19**,** 311-320.

ORTEGA, F. J., VAZQUEZ-MARTIN, A., MORENO-NAVARRETE, J. M., BASSOLS, J., RODRIGUEZ-HERMOSA, J., GIRONES, J., RICART, W., PERAL, B., TINAHONES, F. J., FRUHBECK, G., MENENDEZ, J. A. & FERNANDEZ-REAL, J. M. 2010. Thyroid hormone responsive Spot 14 increases during differentiation of human adipocytes and its expression is down-regulated in obese subjects. *Int J Obes,* 34**,** 487-499.

OSU. 2000a. *Breeds of Livestock - Hereford Cattle* [Online]. Oklahoma, USA: Department of Animal Science, Oklahoma State University. [Accessed 12 June 2015 2015].

OSU. 2000b. *Breeds of Livestock - Holstein Cattle* [Online]. Oklahoma, USA: Department of Animals Science, Oklahoma State University. Available: http://www.ansi.okstate.edu/breeds/cattle/holstein [Accessed 26 May 2015 2015].

PALMQUIST, D. L. 2006. Milk Fat: Origin of Fatty Acids and Influence of Nutritional Factors Thereon. *In:* FOX, P. F. & MCSWEENEY, P. L. H. (eds.) *Advanced Dairy Chemistry Volume 2 Lipids.* Springer US.

PAOLI, A., BOSCO, G., CAMPORESI, E. M. & MANGAR, D. 2015. Ketosis, ketogenic diet and food intake control: a complex relationship. *Frontiers in Psychology,* 6**,** 27.

PAPE-ZAMBITO, D. A., MAGLIARO, A. L. & KENSINGER, R. S. 2008. 17Beta-estradiol and estrone concentrations in plasma and milk during bovine pregnancy. *J Dairy Sci,* 91**,** 127-35.

PAREDES, S. P., KALBE, C., JANSMAN, A. J., VERSTEGEN, M. W., VAN HEES, H. M., LOSEL, D., GERRITS, W. J. & REHFELDT, C. 2013. Predicted high-

performing piglets exhibit more and larger skeletal muscle fibers. *J Anim Sci,* 91**,** 5589-98.
PARK, C. S., CHOI, I. & PARK, Y. S. 2013. Sexual maturation may affect the levels of n-6 PUFA in muscle tissues of male mice. *Journal of Animal Science and Technology* 55**,** 147-153.
PARKER, B., NOAKES, M., LUSCOMBE, N. & CLIFTON, P. 2002. Effect of a High-Protein, High–Monounsaturated Fat Weight Loss Diet on Glycemic Control and Lipid Levels in Type 2 Diabetes. *Diabetes Care,* 25**,** 425-430.
PASZKIEWICZ, K. & STUDHOLME, D. J. 2010. De novo assembly of short sequence reads. *Briefings in Bioinformatics*.
PATEL, O. V., TAKENOUCHI, N., TAKAHASHI, T., HIRAKO, M., SASAKI, N. & DOMEKI, I. 1999. Plasma oestrone and oestradiol concentrations throughout gestation in cattle: relationship to stage of gestation and fetal number. *Res Vet Sci,* 66**,** 129-33.
PEARSON, W. R. & SIERK, M. L. 2005. The limits of protein sequence comparison? *Curr Opin Struct Biol,* 15**,** 254-60.
PEREZ-PEREZ, A., SANCHEZ-JIMENEZ, F., MAYMO, J., DUENAS, J. L., VARONE, C. & SANCHEZ-MARGALET, V. 2015. Role of leptin in female reproduction. *Clin Chem Lab Med,* 53**,** 15-28.
PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* 29**,** e45.
PFUHL, R., BELLMANN, O., KÜHN, C., TEUSCHER, F., ENDER, K. & WEGNER, J. 2007. Beef versus dairy cattle: a comparison of feed conversion, carcass composition and meat quality. *Arch. Anim. Breed.,* 50 59–70
PHE 2014. Tuberculosis in the UK: 2014 report. London, UK: Public Health England.
PHILLIPS, C. J. C. 2010. Cattle production system. *Principles of cattle production.* Wallingford: CABI Publishing.
PHINNEY, D. G. & PROCKOP, D. J. 2007. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells,* 25**,** 2896-902.
PINTO, J. R., GOMES, A. V., JONES, M. A., LIANG, J., NGUYEN, S., MILLER, T., PARVATIYAR, M. S. & POTTER, J. D. 2012. The functional properties of human slow skeletal troponin T isoforms in cardiac muscle regulation. *J Biol Chem,* 287**,** 37362-70.
PLAIN, R. L. & LAWRENCE, J. D. 2003. Swine production. *Vet Clin North Am Food Anim Pract,* 19**,** 319-37.
PLANTE, B. J., KANNAN, A., BAGCHI, M. K., YUAN, L. & YOUNG, S. L. 2009. Cyclic regulation of transcription factor C/EBP beta in human endometrium. *Reprod Biol Endocrinol,* 7**,** 15.
POISSONNET, C. M., BURDI, A. R. & BOOKSTEIN, F. L. 1983. Growth and development of human adipose tissue during early gestation. *Early Hum Dev,* 8**,** 1-11.
POISSONNET, C. M., BURDI, A. R. & GARN, S. M. 1984. The chronology of adipose tissue appearance and distribution in the human fetus. *Early Human Development,* 10**,** 1-11.
POND, W. G. 1991. Of pigs and people. Stoneham, MA, USA: Butterworth-Heinemann.
POND, W. G. & MERSMANN, H. J. 2001. General characteristics. *In:* POND, W. G. & MERSMANN, H. J. (eds.) *Biology of the domestic pig.* Ithaca, New York, USA: Cornell University Press.
POPE, M., BUDGE, H. & SYMONDS, M. E. 2014. The developmental transition of ovine adipose tissue through early life. *Acta Physiol (Oxf),* 210**,** 20-30.
PORTER, V. 1991a. Britain, Ireland and the Channel Islands. *Cattle - The handbook to the breeds of the world.* London: Christopher Helm.

PORTER, V. 1991b. The aurochs. *Cattle - A handbook to the breeds of the world.* London: Christopher Helm.
PORTER, V. 1991c. The development of breeds. *Cattle - A handbook to the breeds of the world.* London: Christopher Helm.
PORTER, V. 1993a. The development of breeds. *Pigs: a handbook to the breeds of the world.* East Sussex, UK.: Helm Information Ltd.
PORTER, V. 1993b. The wild pigs. *Pigs: A handbook to the breeds of the world* East Sussex, UK: Helm Information Ltd.
POSCH, M. G., WALDMULLER, S., MULLER, M., SCHEFFOLD, T., FOURNIER, D., ANDRADE-NAVARRO, M. A., DE GEETER, B., GUILLAUMONT, S., DAUPHIN, C., YOUSSEFF, D., SCHMITT, K. R., PERROT, A., BERGER, F., HETZER, R., BOUVAGNET, P. & OZCELIK, C. 2011. Cardiac alpha-myosin (MYH6) is the predominant sarcomeric disease gene for familial atrial septal defects. *PLoS One,* 6**,** e28872.
POWELL, S. E. & ABERLE, E. D. 1980. Effects of birth weight on growth and carcass composition of swine. *J Anim Sci,* 50**,** 860-8.
PRASAD, P., TIWARI, A. K., KUMAR, K. M., AMMINI, A. C., GUPTA, A., GUPTA, R. & THELMA, B. K. 2010. Association analysis of ADPRT1, AKR1B1, RAGE, GFPT2 and PAI-1 gene polymorphisms with chronic renal insufficiency among Asian Indians with type-2 diabetes. *BMC Med Genet,* 11**,** 52.
PROCTOR, C. M., FREEMAN, E. W. & BROWN, J. L. 2010. Results of a second survey to assess the reproductive status of female Asian and African elephants in North America. *Zoo Biol,* 29**,** 127-39.
PROKOPEC, S. D., WATSON, J. D., WAGGOTT, D. M., SMITH, A. B., WU, A. H., OKEY, A. B., POHJANVIRTA, R. & BOUTROS, P. C. 2013. Systematic evaluation of medium-throughput mRNA abundance platforms. *Rna,* 19**,** 51-62.
PRZYBYT, E., KRENNING, G., BRINKER, M. G. & HARMSEN, M. C. 2013. Adipose stromal cells primed with hypoxia and inflammation enhance cardiomyocyte proliferation rate in vitro through STAT3 and Erk1/2. *J Transl Med,* 11**,** 39.
PUIGSERVER, P. & SPIEGELMAN, B. M. 2003. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev,* 24**,** 78-90.
QUESNEL, H. 2009. Nutritional and lactational effects on follicular development in the pig. *Soc Reprod Fertil Suppl,* 66**,** 121-34.
QUINIOU, N., NOBLET, J., VAN MILGEN, J. & DOURMAD, J. Y. 1995. Effect of energy intake on performance, nutrient and tissue gain and protein and energy utilization in growing boars. *Animal Science,* 61**,** 133-143.
RAHBEK, C. 1993. Captive breeding—a useful tool in the preservation of biodiversity? *Biodiversity & Conservation,* 2**,** 426-437.
RAMIREZ, O., OJEDA, A., TOMAS, A., GALLARDO, D., HUANG, L. S., FOLCH, J. M., CLOP, A., SANCHEZ, A., BADAOUI, B., HANOTTE, O., GALMAN-OMITOGUN, O., MAKUZA, S. M., SOTO, H., CADILLO, J., KELLY, L., CHO, I. C., YEGHOYAN, S., PEREZ-ENCISO, M. & AMILLS, M. 2009. Integrating Y-chromosome, mitochondrial, and autosomal data to analyze the origin of pig breeds. *Mol Biol Evol,* 26**,** 2061-72.
RAMOS, M. P., CRESPO-SOLANS, M. D., DEL CAMPO, S., CACHO, J. & HERRERA, E. 2003. Fat accumulation in the rat during early pregnancy is modulated by enhanced insulin responsiveness. *American Journal of Physiology - Endocrinology and Metabolism,* 285**,** E318-E328.
RAMSAY, T. G., YAN, X. & MORRISON, C. 1998. The obesity gene in swine: sequence and expression of porcine leptin. *J Anim Sci,* 76**,** 484-90.
RANDOLPH, S. & STILES, D. 2011. *Elephant meat trade in Central Africa: Cameroon case study,* Gland, Switzerland, IUCN.

RBST 2011. Rare Breeds Survival Trust fact sheet: Large White. Warwickshire, UK: Rare Breeds Survival Trust.
RBST. 2015. *Aberdeen Angus (Native)* [Online]. Kenilworth, Warwickshire: Rare Breed Survival Trust. [Accessed 21 November 2015].
REBUFFE-SCRIVE, M., ANDERSSON, B., OLBE, L. & BJORNTORP, P. 1989. Metabolism of adipose tissue in intraabdominal depots of nonobese men and women. *Metabolism,* 38**,** 453-8.
REBUFFE-SCRIVE, M., BRONNEGARD, M., NILSSON, A., ELDH, J., GUSTAFSSON, J. A. & BJORNTORP, P. 1990. Steroid hormone receptors in human adipose tissues. *J Clin Endocrinol Metab,* 71**,** 1215-9.
REHFELDT, C. & KUHN, G. 2006. Consequences of birth weight for postnatal growth performance and carcass quality in pigs as related to myogenesis. *J Anim Sci,* 84 Suppl**,** E113-23.
REN, M. Q., WEGNER, J., BELLMANN, O., BROCKMANN, G. A., SCHNEIDER, F., TEUSCHER, F. & ENDER, K. 2002. Comparing mRNA levels of genes encoding leptin, leptin receptor, and lipoprotein lipase between dairy and beef cattle. *Domestic Animal Endocrinology,* 23**,** 371-381.
REPPERT, S. M. & WEAVER, D. R. 2002. Coordination of circadian timing in mammals. *Nature,* 418**,** 935-41.
RHEE, S. Y., WOOD, V., DOLINSKI, K. & DRAGHICI, S. 2008. Use and misuse of the gene ontology annotations. *Nat Rev Genet,* 9**,** 509-15.
RIBOT, J., FELIPE, F., BONET, M. L. & PALOU, A. 2001. Changes of adiposity in response to vitamin A status correlate with changes of PPAR gamma 2 expression. *Obes Res,* 9**,** 500-9.
RICHARD, D. & TRAYHURN, P. 1985. Energetic efficiency during pregnancy in mice fed ad libitum or pair-fed to the normal energy intake of unmated animals. *The Journal of nutrition,* 115**,** 593.
ROBINSON, M. D., MCCARTHY, D. J. & SMYTH, G. K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics,* 26**,** 139-40.
ROBINSON, M. D. & OSHLACK, A. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol,* 11**,** R25.
ROCA, A. L., GEORGIADIS, N., PECON-SLATTERY, J. & O'BRIEN, S. J. 2001. Genetic Evidence for Two Species of Elephant in Africa. *Science,* 293**,** 1473-1477.
ROCHE, H. M. 1999. Unsaturated fatty acids. *Proceedings of the Nutrition Society,* 58**,** 397-401.
ROCHE, J. R., FRIGGENS, N. C., KAY, J. K., FISHER, M. W., STAFFORD, K. J. & BERRY, D. P. 2009. Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. *J Dairy Sci,* 92**,** 5769-801.
ROSEN, E. D., HSU, C. H., WANG, X., SAKAI, S., FREEMAN, M. W., GONZALEZ, F. J. & SPIEGELMAN, B. M. 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev,* 16**,** 22-6.
ROSEN, E. D. & MACDOUGALD, O. A. 2006. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol,* 7**,** 885-96.
ROSEN, E. D., SARRAF, P., TROY, A. E., BRADWIN, G., MOORE, K., MILSTONE, D. S., SPIEGELMAN, B. M. & MORTENSEN, R. M. 1999. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell,* 4**,** 611-7.
ROSEN, E. D. & SPIEGELMAN, B. M. 2006. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature,* 444**,** 847-53.
ROSEN, E. D., WALKEY, C. J., PUIGSERVER, P. & SPIEGELMAN, B. M. 2000. Transcriptional regulation of adipogenesis. *Genes Dev,* 14**,** 1293-307.

ROURKE, J. L., DRANSE, H. J. & SINAL, C. J. 2013. Towards an integrative approach to understanding the role of chemerin in human health and disease. *Obes Rev,* 14**,** 245-62.

ROYAL, M., MANN, G. E. & FLINT, A. P. F. 2000. Strategies for Reversing the Trend Towards Subfertility in Dairy Cattle. *The Veterinary Journal,* 160**,** 53-60.

RUHL, C. E., HARRIS, T. B., DING, J., GOODPASTER, B. H., KANAYA, A. M., KRITCHEVSKY, S. B., SIMONSICK, E. M., TYLAVSKY, F. A., EVERHART, J. E. & FOR THE HEALTH, A. B. C. S. 2007. Body mass index and serum leptin concentration independently estimate percentage body fat in older adults. *The American Journal of Clinical Nutrition,* 85**,** 1121-1126.

SAHU, A. 2003. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. *Frontiers in Neuroendocrinology,* 24**,** 225-253.

SAID, M. Y., CHUNGE, R. N., CRAIG, G. C., THOULESS, C. R., BARNES, R. F. W. & DUBLIN, H. T. 1995. *African Elephant Database 1995,* Gland,Switzerland IUCN.

SAKURAI, H., ERA, T., JAKT, L. M., OKADA, M., NAKAI, S. & NISHIKAWA, S. 2006. In vitro modeling of paraxial and lateral mesoderm differentiation reveals early reversibility. *Stem Cells,* 24**,** 575-86.

SALANS, L. B., CUSHMAN, S. W. & WEISMANN, R. E. 1973. Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients. *J Clin Invest,* 52**,** 929-41.

SALDANHA, A. J. 2004. Java Treeview—extensible visualization of microarray data. *Bioinformatics,* 20**,** 3246-3248.

SAMSOM, M. L., MORRISON, S., MASALA, N., SULLIVAN, B. D., SULLIVAN, D. A., SHEARDOWN, H. & SCHMIDT, T. A. 2014. Characterization of full-length recombinant human Proteoglycan 4 as an ocular surface boundary lubricant. *Exp Eye Res,* 127**,** 14-9.

SANCHEZ-GURMACHES, J. & GUERTIN, D. A. 2014. Adipocyte lineages: Tracing back the origins of fat. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1842**,** 340-351.

SANDERS, W. J., KAPPELMAN, J. & RASMUSSEN, D. T. 2004. New large-bodied mammals from the late Oligocene site of Chilga, Ethiopia. *Acta Palaeontol. Pol. ,* 49**,** 365-392

SANFORD, G. M., LUTTERSCHMIDT, W. I. & HUTCHISON, V. H. 2002. The Comparative Method Revisited. *BioScience,* 52**,** 830-836.

SANTOS, R. L., ZHANG, S., TSOLIS, R. M., KINGSLEY, R. A., ADAMS, L. G. & BAUMLER, A. J. 2001. Animal models of Salmonella infections: enteritis versus typhoid fever. *Microbes Infect,* 3**,** 1335-44.

SATTAR, N., GREER, I. A., PIRWANI, I., GIBSON, J. & WALLACE, A. M. 1998. Leptin levels in pregnancy: marker for fat accumulation and mobilization? *Acta Obstet Gynecol Scand,* 77**,** 278-83.

SATYANARAYANA, A., KLARMANN, K. D., GAVRILOVA, O. & KELLER, J. R. 2012. Ablation of the transcriptional regulator Id1 enhances energy expenditure, increases insulin sensitivity, and protects against age and diet induced insulin resistance, and hepatosteatosis. *Faseb j,* 26**,** 309-23.

SAUNDERS, W. L. & YOUNG, G. D. 1985. An experimental study of the effect of the presence or absence of living visual aids in high school biology classrooms upon attitudes toward science and biology achievement. *Journal of Research in Science Teaching,* 22**,** 619-629.

SCHARRER, E. 1999. Control of food intake by fatty acid oxidation and ketogenesis. *Nutrition,* 15**,** 704-14.

SCHMIDT, M. 1986. Elephants (Proboscidea). *In:* FOWLER, M. (ed.) *Zoo and Wild Animal Medicine.* Philadelphia: W.B. Saunders Co.

SCHNEIDER, J. E. & WADE, G. N. 1990. Decreased availability of metabolic fuels induces anestrus in golden hamsters. *Am J Physiol,* 258**,** R750-5.

SCHRODER, K., WANDZIOCH, K., HELMCKE, I. & BRANDES, R. P. 2009. Nox4 acts as a switch between differentiation and proliferation in preadipocytes. *Arterioscler Thromb Vasc Biol,* 29**,** 239-45.

SCHUPP, M., CRISTANCHO, A. G., LEFTEROVA, M. I., HANNIMAN, E. A., BRIGGS, E. R., STEGER, D. J., QATANANI, M., CURTIN, J. C., SCHUG, J., OCHSNER, S. A., MCKENNA, N. J. & LAZAR, M. A. 2009. Re-expression of GATA2 cooperates with peroxisome proliferator-activated receptor-gamma depletion to revert the adipocyte phenotype. *J Biol Chem,* 284**,** 9458-64.

SCHWARZ, T., KOPYRA, M. & NOWICKI, J. 2008. Physiological mechanisms of ovarian follicular growth in pigs--a review. *Acta Vet Hung,* 56**,** 369-78.

SEKIMOTO, R., FUKUDA, S., MAEDA, N., TSUSHIMA, Y., MATSUDA, K., MORI, T., NAKATSUJI, H., NISHIZAWA, H., KISHIDA, K., KIKUTA, J., MAIJIMA, Y., FUNAHASHI, T., ISHII, M. & SHIMOMURA, I. 2015. Visualized macrophage dynamics and significance of S100A8 in obese fat. *Proceedings of the National Academy of Sciences,* 112**,** E2058-E2066.

SEKIMOTO, R., KISHIDA, K., NAKATSUJI, H., NAKAGAWA, T., FUNAHASHI, T. & SHIMOMURA, I. 2012. High circulating levels of S100A8/A9 complex (calprotectin) in male Japanese with abdominal adiposity and dysregulated expression of S100A8 and S100A9 in adipose tissues of obese mice. *Biochem Biophys Res Commun,* 419**,** 782-9.

SELL, H., LAURENCIKIENE, J., TAUBE, A., ECKARDT, K., CRAMER, A., HORRIGHS, A., ARNER, P. & ECKEL, J. 2009. Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. *Diabetes,* 58**,** 2731-2740.

SEN, A., PRIZANT, H., LIGHT, A., BISWAS, A., HAYES, E., LEE, H. J., BARAD, D., GLEICHER, N. & HAMMES, S. R. 2014. Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression. *Proc Natl Acad Sci U S A,* 111**,** 3008-13.

SETHI, N., MONTEAGUDO, M. C., KOSHLAND, D., HOGAN, E. & BURKE, D. J. 1991. The CDC20 gene product of Saccharomyces cerevisiae, a beta-transducin homolog, is required for a subset of microtubule-dependent cellular processes. *Molecular and Cellular Biology,* 11**,** 5592-5602.

SEUFERT, J. 2004. Leptin effects on pancreatic beta-cell gene expression and function. *Diabetes,* 53 Suppl 1**,** S152-8.

SHAN, T., WANG, Y., GUO, J., CHU, X., LIU, J. & XU, Z. 2008. The body weight-related differences of leptin and neuropeptide Y (NPY) gene expression in pigs. *Asian-Aust. J. Anim. Sci.,* 21**,** 161 - 166.

SHI, H., NORMAN, A. W., OKAMURA, W. H., SEN, A. & ZEMEL, M. B. 2002. 1alpha,25-dihydroxyvitamin D3 inhibits uncoupling protein 2 expression in human adipocytes. *Faseb j,* 16**,** 1808-10.

SHIMADA, M. & TERADA, T. 2002. FSH and LH induce progesterone production and progesterone receptor synthesis in cumulus cells: a requirement for meiotic resumption in porcine oocytes. *Mol Hum Reprod,* 8**,** 612-8.

SHIMIZU, H., SHIMOMURA, Y., HAYASHI, R., OHTANI, K., SATO, N., FUTAWATARI, T. & MORI, M. 1997a. Serum leptin concentration is associated with total body fat mass, but not abdominal fat distribution. *Int J Obes Relat Metab Disord,* 21**,** 536-41.

SHIMIZU, H., SHIMOMURA, Y., NAKANISHI, Y., FUTAWATARI, T., OHTANI, K., SATO, N. & MORI, M. 1997b. Estrogen increases in vivo leptin production in rats and human subjects. *J Endocrinol,* 154**,** 285-92.

SHIMODAIRA, H. 2002. An Approximately Unbiased Test of Phylogenetic Tree Selection. *Systematic Biology,* 51**,** 492-508.

SHIMODAIRA, H. 2004. Approximately unbiased tests of regions using multistep-multiscale bootstrap resampling. 2616-2641.
SHIMOMURA, I., SHIMANO, H., HORTON, J. D., GOLDSTEIN, J. L. & BROWN, M. S. 1997. Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *The Journal of Clinical Investigation,* 99**,** 838-845.
SHIRLEY, J. 2011. A comparative study would begin to settle debate over Hereford genetics. *Hereford Breed Journal***,** 170 - 171
SHOSHANI, J. 2006. Taxonomy, classification, history, and evolution of elephants. *In:* FOWLER, M. E. & MIKOTA, S. K. (eds.) *Elephant biology, medicine, and surgery.* Iowa, USA: Blackwell Publishing.
SIAWRYS, G. & SMOLINSKA, N. 2013. In vitro effects of luteinizing hormone, progesterone and oestradiol-17beta on leptin gene expression and leptin secretion by porcine luteal cells obtained in early pregnancy. *J Physiol Pharmacol,* 64**,** 513-20.
SIMOPOULOS, A. P. 1991. Omega-3 fatty acids in health and disease and in growth and development. *Am J Clin Nutr,* 54**,** 438-63.
SIMOPOULOS, A. P. 1999. Essential fatty acids in health and chronic disease. *The American Journal of Clinical Nutrition,* 70**,** 560s-569s.
SIMPSON, E. R. 2003. Sources of estrogen and their importance. *J Steroid Biochem Mol Biol,* 86**,** 225-30.
SINCLAIR, K. D. 2010. Declining fertility, insulin resistance and fatty acid metabolism in dairy cows: Developmental consequences for the oocyte and pre-implantation embryo *Acta Scientiae Veterinariae,* 38(Supl 2)**,** s545-s557.
SINGH, A., WIRTZ, M., PARKER, N., HOGAN, M., STRAHLER, J., MICHAILIDIS, G., SCHMIDT, S., VIDAL-PUIG, A., DIANO, S., ANDREWS, P., BRAND, M. D. & FRIEDMAN, J. 2009. Leptin-mediated changes in hepatic mitochondrial metabolism, structure, and protein levels. *Proceedings of the National Academy of Sciences,* 106**,** 13100-13105.
SLY, W. S. & HU, P. Y. 1995. Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu Rev Biochem,* 64**,** 375-401.
SMEDMAN, A. E. M., GUSTAFSSON, I.-B., BERGLUND, L. G. T. & VESSBY, B. O. H. 1999. Pentadecanoic acid in serum as a marker for intake of milk fat: relations between intake of milk fat and metabolic risk factors. *The American Journal of Clinical Nutrition,* 69**,** 22-29.
SMEMO, S., TENA, J. J., KIM, K. H., GAMAZON, E. R., SAKABE, N. J., GOMEZ-MARIN, C., ANEAS, I., CREDIDIO, F. L., SOBREIRA, D. R., WASSERMAN, N. F., LEE, J. H., PUVIINDRAN, V., TAM, D., SHEN, M., SON, J. E., VAKILI, N. A., SUNG, H. K., NARANJO, S., ACEMEL, R. D., MANZANARES, M., NAGY, A., COX, N. J., HUI, C. C., GOMEZ-SKARMETA, J. L. & NOBREGA, M. A. 2014. Obesity-associated variants within FTO form long-range functional connections with IRX3. *Nature,* 507**,** 371-5.
SMET, S. D., WEBB, E. C., CLAEYS, E., UYTTERHAEGEN, L. & DEMEYER, D. I. 2000. Effect of dietary energy and protein levels on fatty acid composition of intramuscular fat in double-muscled Belgian Blue bulls. *Meat Science,* 56**,** 73-79.
SMITH, B. & HUTCHINS, M. 2000. The value of captive breeding programmes to field conservation: elephant as an example. *Pachyderm,* 28**,** 101-109
SMITH, S. 1994. The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. *Faseb j,* 8**,** 1248-59.
SMITH, S., WITKOWSKI, A. & JOSHI, A. K. 2003. Structural and functional organization of the animal fatty acid synthase. *Prog Lipid Res,* 42**,** 289-317.
SMITH, U. 1985. Regional differences in adipocyte metabolism and possible consequences in vivo. *Int J Obes,* 9 Suppl 1**,** 145-8.

SMOLINSKA, N., KAMINSKI, T., SIAWRYS, G. & PRZALA, J. 2013. Expression of leptin and its receptor genes in the ovarian follicles of cycling and early pregnant pigs. *Animal,* 7**,** 109-17.

SOEDE, N. M., LANGENDIJK, P. & KEMP, B. 2011. Reproductive cycles in pigs. *Anim Reprod Sci,* 124**,** 251-8.

SPALDING, K. L., ARNER, E., WESTERMARK, P. O., BERNARD, S., BUCHHOLZ, B. A., BERGMANN, O., BLOMQVIST, L., HOFFSTEDT, J., NASLUND, E., BRITTON, T., CONCHA, H., HASSAN, M., RYDEN, M., FRISEN, J. & ARNER, P. 2008. Dynamics of fat cell turnover in humans. *Nature,* 453**,** 783-7.

SPURLOCK, M. E., FRANK, G. R., CORNELIUS, S. G., JI, S., WILLIS, G. M. & BIDWELL, C. A. 1998. Obese gene expression in porcine adipose tissue is reduced by food deprivation but not by maintenance or submaintenance intake. *J Nutr,* 128**,** 677-82.

STEINGRIMSDOTTIR, L., GREENWOOD, M. R. & BRASEL, J. A. 1980. Effect of pregnancy, lactation and a high-fat diet on adipose tissue in Osborne-Mendel rats. *J Nutr,* 110**,** 600-9.

STEPIEN, M., GAUDICHON, C., FROMENTIN, G., EVEN, P., TOME, D. & AZZOUT-MARNICHE, D. 2011. Increasing protein at the expense of carbohydrate in the diet down-regulates glucose utilization as glucose sparing effect in rats. *PLoS One,* 6**,** e14664.

STEPPAN, C. M., BAILEY, S. T., BHAT, S., BROWN, E. J., BANERJEE, R. R., WRIGHT, C. M., PATEL, H. R., AHIMA, R. S. & LAZAR, M. A. 2001. The hormone resistin links obesity to diabetes. *Nature,* 409**,** 307-12.

STRABLE, M. S. & NTAMBI, J. M. 2010. Genetic control of de novo lipogenesis: role in diet-induced obesity. *Critical reviews in biochemistry and molecular biology,* 45**,** 199-214.

STRAUSS, R. S. 1999. Comparison of serum concentrations of α-tocopherol and β-carotene in a cross-sectional sample of obese and nonobese children (NHANES III). *The Journal of Pediatrics,* 134**,** 160-165.

STUEBE, A. M. & RICH-EDWARDS, J. W. 2009. The Reset Hypothesis: Lactation and Maternal Metabolism. *American journal of perinatology,* 26**,** 81-88.

SUGA, A., HIRANO, T., INOUE, S., TSUJI, M., OSAKA, T., NAMBA, Y., MIURA, M. & ADACHI, M. 1999. Plasma leptin levels and triglyceride secretion rates in VMH-lesioned obese rats: a role of adiposity. *Am J Physiol,* 276**,** E650-7.

SUH, Y. H., KIM, W. H., MOON, C., HONG, Y. H., EUN, S. Y., LIM, J. H., CHOI, J. S., SONG, J. & JUNG, M. H. 2005a. Ectopic expression of Neuronatin potentiates adipogenesis through enhanced phosphorylation of cAMP-response element-binding protein in 3T3-L1 cells. *Biochem Biophys Res Commun.* United States.

SUH, Y. H., KIM, Y., BANG, J. H., CHOI, K. S., LEE, J. W., KIM, W. H., OH, T. J., AN, S. & JUNG, M. H. 2005b. Analysis of gene expression profiles in insulin-sensitive tissues from pre-diabetic and diabetic Zucker diabetic fatty rats. *J Mol Endocrinol,* 34**,** 299-315.

SUN, T., HAYAKAWA, K., BATEMAN, K. S. & FRASER, M. E. 2010. Identification of the citrate-binding site of human ATP-citrate lyase using X-ray crystallography. *J Biol Chem,* 285**,** 27418-28.

SUTHERLAND, E. W. & ROBISON, G. A. 1969. The Role of Cyclic AMP in the Control of Carbohydrate Metabolism. *Diabetes,* 18**,** 797-819.

SUZUKI, R. & SHIMODAIRA, H. 2006. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics,* 22**,** 1540-1542.

SUZUKI, R., TOBE, K., AOYAMA, M., SAKAMOTO, K., OHSUGI, M., KAMEI, N., NEMOTO, S., INOUE, A., ITO, Y., UCHIDA, S., HARA, K., YAMAUCHI, T., KUBOTA, N., TERAUCHI, Y. & KADOWAKI, T. 2005. Expression of DGAT2

in white adipose tissue is regulated by central leptin action. *J Biol Chem,* 280**,** 3331-7.
SWIERCZYNSKI, J. 2006. Leptin and age-related down-regulation of lipogenic enzymes genes expression in rat white adipose tissue. *J Physiol Pharmacol,* 57 Suppl 6**,** 85-102.
TAGA, H., CHILLIARD, Y., MEUNIER, B., CHAMBON, C., PICARD, B., ZINGARETTI, M. C., CINTI, S. & BONNET, M. 2012a. Cellular and molecular large-scale features of fetal adipose tissue: is bovine perirenal adipose tissue brown? *J Cell Physiol,* 227**,** 1688-700.
TAGA, H., CHILLIARD, Y., PICARD, B., ZINGARETTI, M. C. & BONNET, M. 2012b. Foetal bovine intermuscular adipose tissue exhibits histological and metabolic features of brown and white adipocytes during the last third of pregnancy. *Animal,* 6**,** 641-9.
TAKASE, S., ONG, D. E. & CHYTIL, F. 1986. Transfer of retinoic acid from its complex with cellular retinoic acid-binding protein to the nucleus. *Arch Biochem Biophys,* 247**,** 328-34.
TANNER, S. M., LI, Z., PERKO, J. D., ONER, C., CETIN, M., ALTAY, C., YURTSEVER, Z., DAVID, K. L., FAIVRE, L., ISMAIL, E. A., GRASBECK, R. & DE LA CHAPELLE, A. 2005. Hereditary juvenile cobalamin deficiency caused by mutations in the intrinsic factor gene. *Proc Natl Acad Sci U S A,* 102**,** 4130-3.
TATMAN, W. R., JUDKINS, M. B., DUNN, T. G. & MOSS, G. E. 1990. Luteinizing hormone in nutrient-restricted ovariectomized ewes. *J Anim Sci,* 68**,** 1097-102.
TCHKONIA, T., LENBURG, M., THOMOU, T., GIORGADZE, N., FRAMPTON, G., PIRTSKHALAVA, T., CARTWRIGHT, A., CARTWRIGHT, M., FLANAGAN, J., KARAGIANNIDES, I., GERRY, N., FORSE, R. A., TCHOUKALOVA, Y., JENSEN, M. D., POTHOULAKIS, C. & KIRKLAND, J. L. 2007. Identification of depot-specific human fat cell progenitors through distinct expression profiles and developmental gene patterns. *Am J Physiol Endocrinol Metab,* 292**,** E298-307.
TCHKONIA, T., THOMOU, T., ZHU, Y., KARAGIANNIDES, I., POTHOULAKIS, C., JENSEN, M. D. & KIRKLAND, J. L. 2013. Mechanisms and metabolic implications of regional differences among fat depots. *Cell Metab,* 17**,** 644-56.
THATCHER, W. W., BINELLI, M., BURKE, J., STAPLES, C. R., AMBROSE, J. D. & COELHO, S. 1997. Antiluteolytic signals between the conceptus and endometrium. *Theriogenology,* 47**,** 131-140.
THECATTLESITE. 2014. *Hereford* [Online]. Sheffield, UK: 5m Publishing. [Accessed 12 June 2015 2015].
THOEN, C., LOBUE, P. & DE KANTOR, I. 2006. The importance of Mycobacterium bovis as a zoonosis. *Veterinary Microbiology,* 112**,** 339-345.
THOMSON, J. M., STOTHARD, P. & MCNAMARA, J. P. 2013. Transcriptome profile comparison between beef and dairy adipose pooled mRNA reveals differences. *In:* OLTJEN, J., KEBREAB, E. & LAPIERRE, H. (eds.) *Energy and protein metabolism and nutrition in sustainable animal production.* Wageningen Academic Publishers.
THONGTIP, N., MAHASAWANGKUL, S., THITARAM, C., PONGSOPAVIJITR, P., KORNKAEWRAT, K., PINYOPUMMIN, A., ANGKAWANISH, T., JANSITTIWATE, S., RUNGSRI, R., BOONPRASERT, K., WONGKALASIN, W., HOMKONG, P., DEJCHAISRI, S., WAJJWALKU, W. & SAIKHUN, K. 2009. Successful artificial insemination in the Asian elephant (Elephas maximus) using chilled and frozen-thawed semen. *Reproductive Biology and Endocrinology : RB&E,* 7**,** 75-75.
THONNEY, M. L. 1987. Growth, feed efficiency and variation of individually fed Angus, polled Hereford and Holstein steers. *J Anim Sci,* 65**,** 1-8.

TOMARU, T., STEGER, D. J., LEFTEROVA, M. I., SCHUPP, M. & LAZAR, M. A. 2009. Adipocyte-specific expression of murine resistin is mediated by synergism between peroxisome proliferator-activated receptor gamma and CCAAT/enhancer-binding proteins. *J Biol Chem,* 284**,** 6116-25.

TONG, L. 2005. Acetyl-coenzyme A carboxylase: crucial metabolic enzyme and attractive target for drug discovery. *Cell Mol Life Sci,* 62**,** 1784-803.

TONTONOZ, P., HU, E. & SPIEGELMAN, B. M. 1994. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell,* 79**,** 1147-1156.

TONTONOZ, P. & SPIEGELMAN, B. M. 2008. Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem,* 77**,** 289-312.

TRAISTER, R. S., UVALLE, C. E., HAWKINS, G. A., MEYERS, D. A., BLEECKER, E. R. & WENZEL, S. E. 2015. Phenotypic and genotypic association of epithelial IL1RL1 to human TH2-like asthma. *J Allergy Clin Immunol,* 135**,** 92-9.

TRASINO, S. E., BENOIT, Y. D. & GUDAS, L. J. 2015. Vitamin A deficiency causes hyperglycemia and loss of pancreatic beta-cell mass. *J Biol Chem,* 290**,** 1456-73.

TRAYHURN, P. & BEATTIE, J. H. 2001. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc,* 60**,** 329-39.

TRAYHURN, P., HOGGARD, N., MERCER, J. G. & RAYNER, D. V. 1999. Leptin: fundamental aspects. *Int J Obes Relat Metab Disord,* 23 Suppl 1**,** 22-8.

TRAYHURN, P., TEMPLE, N. J. & VAN AERDE, J. 1989. Evidence from immunoblotting studies on uncoupling protein that brown adipose tissue is not present in the domestic pig. *Can J Physiol Pharmacol,* 67**,** 1480-5.

TREMBLAY, K., VIGNEAULT, C., MCGRAW, S., MORIN, G. & SIRARD, M. A. 2006. Identification and characterization of a novel bovine oocyte-specific secreted protein gene. *Gene,* 375**,** 44-53.

TRIPATHY, S., LYTLE, K. A., STEVENS, R. D., BAIN, J. R., NEWGARD, C. B., GREENBERG, A. S., HUANG, L. S. & JUMP, D. B. 2014. Fatty acid elongase-5 (Elovl5) regulates hepatic triglyceride catabolism in obese C57BL/6J mice. *J Lipid Res,* 55**,** 1448-1464.

TRIPATHY, S., TORRES-GONZALEZ, M. & JUMP, D. B. 2010. Elevated hepatic fatty acid elongase-5 activity corrects dietary fat-induced hyperglycemia in obese C57BL/6J mice. *J Lipid Res,* 51**,** 2642-54.

TROY, C. S., MACHUGH, D. E., BAILEY, J. F., MAGEE, D. A., LOFTUS, R. T., CUNNINGHAM, P., CHAMBERLAIN, A. T., SYKES, B. C. & BRADLEY, D. G. 2001. Genetic evidence for Near-Eastern origins of European cattle. *Nature,* 410**,** 1088-1091.

TSAI, S. H., CHANG, E. Y., CHANG, Y. C., HEE, S. W., TSAI, Y. C., CHANG, T. J. & CHUANG, L. M. 2013. Knockdown of RyR3 enhances adiponectin expression through an atf3-dependent pathway. *Endocrinology,* 154**,** 1117-29.

TSAO, T.-S., HUG, C. & LODISH, H. F. 2003. Adipokines: Regulators of Metabolic Integration and Energy Metabolism. *In:* LEROITH, D., OLEFSKY, J. M. & TAYLOR, S. I. (eds.) *Diabetes Mellitus: A Fundamental and Clinical Text.* 3rd ed. Philadelphia, USA: Lippincott Williams and Wilkins.

TURYN, J., MIKA, A., STEPNOWSKI, P. & SWIERCZYNSKI, J. 2012. Unusual increase of Scd1 and Elovl6 expression in rat inguinal adipose tissue. *Central European Journal of Biology,* 7**,** 192-200.

UAB. 2014. *Does this trunk make me look fat? Overweight zoo elephants no laughing matter* [Online]. ScienceDaily. [Accessed 19 July 2015 2015].

UNEP, CITES, IUCN & TRAFFIC 2013. *Elephants in the Dust – The African Elephant Crisis. A Rapid Response Assessment,* Arendal, Norway, GRID-Arendal.

VAIDYANATHAN, G., CISMOWSKI, M. J., WANG, G., VINCENT, T. S., BROWN, K. D. & LANIER, S. M. 2004. The Ras-related protein AGS1/RASD1 suppresses cell growth. *Oncogene,* 23**,** 5858-63.

VALLET, J. L., CHRISTENSON, R. K., TROUT, W. E. & KLEMCKE, H. G. 1998. Conceptus, progesterone, and breed effects on uterine protein secretion in swine. *J Anim Sci,* 76**,** 2657-70.

VAN DER WALT, J. G. & MEYER, J. H. F. 1988. Protein digestion in ruminants. *S Afr J Anim Sci,* 18**,** 30-41

VAN HOUTERT, M. F. J. 1993. The production and metabolism of volatile fatty acids by ruminants fed roughages: A review. *Animal Feed Science and Technology,* 43**,** 189-225.

VAN RHIJN, I., GODFROID, J., MICHEL, A. & RUTTEN, V. 2008. Bovine tuberculosis as a model for human tuberculosis: advantages over small animal models. *Microbes Infect,* 10**,** 711-5.

VARMA, S., BASKARAN, N. & SUKUMAR, R. 2012. Age and sex classification. *Field Key for Elephant Population Estimation and Age and Sex Classification.* Bangalore, India: Karnataka and Centre for Ecological Sciences, Indian Institute of Science.

VELDHORST, M. A., WESTERTERP, K. R., VAN VUGHT, A. J. & WESTERTERP-PLANTENGA, M. S. 2010. Presence or absence of carbohydrates and the proportion of fat in a high-protein diet affect appetite suppression but not energy expenditure in normal-weight human subjects fed in energy balance. *Br J Nutr,* 104**,** 1395-405.

VELDHORST, M. A., WESTERTERP-PLANTENGA, M. S. & WESTERTERP, K. R. 2009. Gluconeogenesis and energy expenditure after a high-protein, carbohydrate-free diet. *Am J Clin Nutr,* 90**,** 519-26.

VERNON, R. G. 2005. Lipid metabolism during lactation: a review of adipose tissue-liver interactions and the development of fatty liver. *J Dairy Res,* 72**,** 460-9.

VIDAL, H. 2001. Gene expression in visceral and subcutaneous adipose tissues. *Ann Med,* 33**,** 547-55.

VIGUERIE, N., MONTASTIER, E., MAORET, J.-J., ROUSSEL, B., COMBES, M., VALLE, C., VILLA-VIALANEIX, N., IACOVONI, J. S., MARTINEZ, J. A., HOLST, C., ASTRUP, A., VIDAL, H., CLÉMENT, K., HAGER, J., SARIS, W. H. M. & LANGIN, D. 2012. Determinants of Human Adipose Tissue Gene Expression: Impact of Diet, Sex, Metabolic Status, and <italic>Cis</italic> Genetic Regulation. *PLoS Genet,* 8**,** e1002959.

VIKMAN, H. L., SAVOLA, J. M., RAASMAJA, A. & OHISALO, J. J. 1996. Alpha 2A-adrenergic regulation of cyclic AMP accumulation and lipolysis in human omental and subcutaneous adipocytes. *Int J Obes Relat Metab Disord,* 20**,** 185-9.

VILARRASA, N., MARAVALL, J., ESTEPA, A., SANCHEZ, R., MASDEVALL, C., NAVARRO, M. A., ALIA, P., SOLER, J. & GOMEZ, J. M. 2007. Low 25-hydroxyvitamin D concentrations in obese women: their clinical significance and relationship with anthropometric and body composition variables. *J Endocrinol Invest,* 30**,** 653-8.

VILLACA CHAVES, G., PEREIRA, S. E., SABOYA, C. J. & RAMALHO, A. 2008. Non-alcoholic fatty liver disease and its relationship with the nutritional status of vitamin A in individuals with class III obesity. *Obes Surg,* 18**,** 378-85.

VINSKY, M. D., NOVAK, S., DIXON, W. T., DYCK, M. K. & FOXCROFT, G. R. 2006. Nutritional restriction in lactating primiparous sows selectively affects female embryo survival and overall litter development. *Reprod Fertil Dev,* 18**,** 347-55.

VLAEMINCK, B., FIEVEZ, V., CABRITA, A. R. J., FONSECA, A. J. M. & DEWHURST, R. J. 2006. Factors affecting odd- and branched-chain fatty acids in milk: A review. *Animal Feed Science and Technology,* 131**,** 389-417.

VODICKA, P., SMETANA, K., JR., DVORANKOVA, B., EMERICK, T., XU, Y. Z., OUREDNIK, J., OUREDNIK, V. & MOTLIK, J. 2005. The miniature pig as an animal model in biomedical research. *Ann N Y Acad Sci,* 1049**,** 161-71.

VOGEL, S., MENDELSOHN, C. L., MERTZ, J. R., PIANTEDOSI, R., WALDBURGER, C., GOTTESMAN, M. E. & BLANER, W. S. 2001. Characterization of a new member of the fatty acid-binding protein family that binds all-trans-retinol. *J Biol Chem,* 276**,** 1353-60.

VOHL, M. C., SLADEK, R., ROBITAILLE, J., GURD, S., MARCEAU, P., RICHARD, D., HUDSON, T. J. & TCHERNOF, A. 2004. A survey of genes differentially expressed in subcutaneous and visceral adipose tissue in men. *Obes Res,* 12**,** 1217-22.

VREELAND, A. C., LEVI, L., ZHANG, W., BERRY, D. C. & NOY, N. 2014. Cellular Retinoic Acid-binding Protein 2 Inhibits Tumor Growth by Two Distinct Mechanisms. *J Biol Chem,* 289**,** 34065-73.

WADE, G. N. & JONES, J. E. 2004. Neuroendocrinology of nutritional infertility. *Am J Physiol Regul Integr Comp Physiol,* 287**,** R1277-96.

WADE, G. N. & SCHNEIDER, J. E. 1992. Metabolic fuels and reproduction in female mammals. *Neuroscience & Biobehavioral Reviews,* 16**,** 235-272.

WADE, G. N., SCHNEIDER, J. E. & LI, H. Y. 1996. Control of fertility by metabolic cues. *Am J Physiol,* 270**,** E1-19.

WALKER, G. 2012. Amino Acid Balance – the next step in cattle feeding technology for maximum genetic expression. Quincy, Illinois, USA ADM Alliance Nutrition, Inc.

WALSH, S. W., WILLIAMS, E. J. & EVANS, A. C. O. 2011. A review of the causes of poor fertility in high milk producing dairy cows. *Animal Reproduction Science,* 123**,** 127-138.

WALTERS, E. M. & PRATHER, R. S. 2013. Advancing swine models for human health and diseases. *Mo Med,* 110**,** 212-5.

WANG, J., DUNCAN, D., SHI, Z. & ZHANG, B. 2013. WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Research,* 41**,** W77-W83.

WANG, M. Y., LEE, Y. & UNGER, R. H. 1999a. Novel form of lipolysis induced by leptin. *J Biol Chem,* 274**,** 17541-4.

WANG, S., WATANABE, T., NORITAKE, J., FUKATA, M., YOSHIMURA, T., ITOH, N., HARADA, T., NAKAGAWA, M., MATSUURA, Y., ARIMURA, N. & KAIBUCHI, K. 2007. IQGAP3, a novel effector of Rac1 and Cdc42, regulates neurite outgrowth. *J Cell Sci,* 120**,** 567-77.

WANG, Y., BARBACIORU, C., HYLAND, F., XIAO, W., HUNKAPILLER, K. L., BLAKE, J., CHAN, F., GONZALEZ, C., ZHANG, L. & SAMAHA, R. R. 2006. Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays. *BMC Genomics,* 7**,** 59.

WANG, Y., BOTOLIN, D., CHRISTIAN, B., BUSIK, J., XU, J. & JUMP, D. B. 2005. Tissue-specific, nutritional, and developmental regulation of rat fatty acid elongases. *J Lipid Res,* 46**,** 706-15.

WANG, Y., JONES VOY, B., URS, S., KIM, S., SOLTANI-BEJNOOD, M., QUIGLEY, N., HEO, Y.-R., STANDRIDGE, M., ANDERSEN, B., DHAR, M., JOSHI, R., WORTMAN, P., TAYLOR, J. W., CHUN, J., LEUZE, M., CLAYCOMBE, K., SAXTON, A. M. & MOUSTAID-MOUSSA, N. 2004. The human fatty acid synthase gene and de novo lipogenesis are coordinately regulated in human adipose tissue. *The Journal of Nutrition,* 134**,** 1032-1038.

WANG, Z., GERSTEIN, M. & SNYDER, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet,* 10**,** 57-63.

WANG, Z. W., ZHOU, Y. T., LEE, Y., HIGA, M., KALRA, S. P. & UNGER, R. H. 1999b. Hyperleptinemia depletes fat from denervated fat tissue. *Biochem Biophys Res Commun,* 260**,** 653-7.

WARD, R. E., WOODWARD, B., OTTER, N. & DORAN, O. 2010. Relationship between the expression of key lipogenic enzymes, fatty acid composition, and intramuscular fat content of Limousin and Aberdeen Angus cattle. *Livestock Science,* 127**,** 22-29.

WASSER, S. K., PAPAGEORGE, S., FOLEY, C. & BROWN, J. L. 1996. Excretory fate of estradiol and progesterone in the African elephant (Loxodonta africana) and patterns of fecal steroid concentrations throughout the estrous cycle. *Gen Comp Endocrinol,* 102**,** 255-62.

WATHES, D. C., ABAYASEKARA, D. R. E. & AITKEN, R. J. 2007. Polyunsaturated Fatty Acids in Male and Female Reproduction. *Biology of Reproduction,* 77**,** 190-201.

WATHES, D. C., BRICKELL, J. S., BOURNE, N. E., SWALI, A. & CHENG, Z. 2008. Factors influencing heifer survival and fertility on commercial dairy farms. *Animal,* 2**,** 1135-43.

WATHES, D. C., CLEMPSON, A. M. & POLLOTT, G. E. 2012. Associations between lipid metabolism and fertility in the dairy cow. *Reproduction, Fertility and Development,* 25**,** 48-61.

WATHES, D. C., POLLOTT, G. E., JOHNSON, K. F., RICHARDSON, H. & COOKE, J. S. 2014. Heifer fertility and carry over consequences for life time production in dairy and beef cattle. *Animal,* 8 Suppl 1**,** 91-104.

WELLEN, K. E., HATZIVASSILIOU, G., SACHDEVA, U. M., BUI, T. V., CROSS, J. R. & THOMPSON, C. B. 2009. ATP-citrate lyase links cellular metabolism to histone acetylation. *Science,* 324**,** 1076-80.

WERNERSSON, R., SCHIERUP, M. H., JORGENSEN, F. G., GORODKIN, J., PANITZ, F., STAERFELDT, H. H., CHRISTENSEN, O. F., MAILUND, T., HORNSHOJ, H., KLEIN, A., WANG, J., LIU, B., HU, S., DONG, W., LI, W., WONG, G. K., YU, J., BENDIXEN, C., FREDHOLM, M., BRUNAK, S., YANG, H. & BOLUND, L. 2005. Pigs in sequence space: a 0.66X coverage pig genome survey based on shotgun sequencing. *BMC Genomics,* 6**,** 70.

WESTERBERG, R., MANSSON, J. E., GOLOZOUBOVA, V., SHABALINA, I. G., BACKLUND, E. C., TVRDIK, P., RETTERSTOL, K., CAPECCHI, M. R. & JACOBSSON, A. 2006. ELOVL3 is an important component for early onset of lipid recruitment in brown adipose tissue. *J Biol Chem,* 281**,** 4958-68.

WESTERTERP-PLANTENGA, M. S. 2003. The significance of protein in food intake and body weight regulation. *Curr Opin Clin Nutr Metab Care,* 6**,** 635-8.

WHITE, B. R., BARNES, J. E. & WHEELER, M. B. 1996. Reproductive Physiology in Chinese Meishan Pigs. *In:* TUMBLESON, M. E. & SCHOOK, L. B. (eds.) *Advances in Swine in Biomedical Research.* Springer US.

WHITE, B. R., GERFEN, R. W., WALTERS, E. M. & WHEELER, M. B. 2000. Comparisons of culture of Chinese Meishan with Yorkshire pig embryos in vitro: effects of protein supplementation and development. *J. Appl. Anim. Res.,* 17**,** 169–184.

WHITE, B. R., LAN, Y. H., MCKEITH, F. K., MCLAREN, D. G., NOVAKOFSKI, J., WHEELER, M. B. & KASSER, T. R. 1993a. Effects of porcine somatotropin on growth and carcass composition of Meishan and Yorkshire barrows. *J Anim Sci,* 71**,** 3226-38.

WHITE, B. R., LAN, Y. H., MCKEITH, F. K., NOVAKOFSKI, J., WHEELER, M. B. & MCLAREN, D. G. 1995. Growth and body composition of Meishan and Yorkshire barrows and gilts. *J Anim Sci,* 73**,** 738-49.

WHITE, B. R., MCLAREN, D. G., DZIUK, P. J. & WHEELER, M. B. 1993b. Age at puberty, ovulation rate, uterine length, prenatal survival and litter size in Chinese Meishan and Yorkshire females. *Theriogenology,* 40**,** 85-97.

WHITE, B. R. & WHEELER, M. B. 1995. Examination of ovulation rate, uterine and fetal interactions, and reproductive age in chinese-meishan, yorkshire, and

reciprocal cross gilts - effects of fetal and maternal genotypes. *Animal Reproduction Science,* 39**,** 147-158.

WHITSON, R. H., TSARK, W., HUANG, T. H. & ITAKURA, K. 2003. Neonatal mortality and leanness in mice lacking the ARID transcription factor Mrf-2. *Biochem Biophys Res Commun,* 312**,** 997-1004.

WICKELMAIER, F. 2003. *An introduction to MDS* [Online]. Aalboarg East, Denmark. : Sound Quality Research Unit, Alaborg University. [Accessed 6 September 2015 2015].

WIESE, R. J. & WILLIS, K. 2006. Population management of zoo elephants. *International Zoo Yearbook,* 40**,** 80-87.

WIGMORE, P. M. & STICKLAND, N. C. 1983. Muscle development in large and small pig fetuses. *J Anat,* 137 (Pt 2)**,** 235-45.

WILDING, J. P., GILBEY, S. G., BAILEY, C. J., BATT, R. A., WILLIAMS, G., GHATEI, M. A. & BLOOM, S. R. 1993. Increased neuropeptide-Y messenger ribonucleic acid (mRNA) and decreased neurotensin mRNA in the hypothalamus of the obese (ob/ob) mouse. *Endocrinology,* 132**,** 1939-44.

WILLIAMS, G. L., AMSTALDEN, M., GARCIA, M. R., STANKO, R. L., NIZIELSKI, S. E., MORRISON, C. D. & KEISLER, D. H. 2002. Leptin and its role in the central regulation of reproduction in cattle. *Domest Anim Endocrinol,* 23**,** 339-49.

WILMUT, I., RITCHIE, W. A., HALEY, C. S., ASHWORTH, C. J. & AITKEN, R. P. 1992. A comparison of rate and uniformity of embryo development in Meishan and European white pigs. *J Reprod Fertil,* 95**,** 45-56.

WILSON, M. E., BIENSEN, N. J., YOUNGS, C. R. & FORD, S. P. 1998. Development of Meishan and Yorkshire littermate conceptuses in either a Meishan or Yorkshire uterine environment to day 90 of gestation and to term. *Biol Reprod,* 58**,** 905-10.

WISE, T., KLINDT, J., HOWARD, H. J., CONLEY, A. J. & FORD, J. J. 2001. Endocrine relationships of Meishan and White composite females after weaning and during the luteal phase of the estrous cycle. *J Anim Sci,* 79**,** 176-187.

WITTEMYER, G., NORTHRUP, J. M., BLANC, J., DOUGLAS-HAMILTON, I., OMONDI, P. & BURNHAM, K. P. 2014. Illegal killing for ivory drives global decline in African elephants. *Proc Natl Acad Sci U S A,* 111**,** 13117-21.

WOLDEN-HANSON, T. 2010. Changes in body composition in response to challenges during aging in rats. *Interdiscip Top Gerontol,* 37**,** 64-83.

WRAY, G. A. & ABOUHEIF, E. 1998. When is homology not homology? *Current Opinion in Genetics & Development,* 8**,** 675-680.

WRONSKA, A., SLEDZINSKI, T., GOYKE, E., LAWNICZAK, A., WIERZBICKI, P. & KMIEC, Z. 2014. Short-term calorie restriction and refeeding differently affect lipogenic enzymes in major white adipose tissue depots of young and old rats. *J Physiol Pharmacol,* 65**,** 117-26.

WU, J., BOSTROM, P., SPARKS, L. M., YE, L., CHOI, J. H., GIANG, A. H., KHANDEKAR, M., VIRTANEN, K. A., NUUTILA, P., SCHAART, G., HUANG, K., TU, H., VAN MARKEN LICHTENBELT, W. D., HOEKS, J., ENERBACK, S., SCHRAUWEN, P. & SPIEGELMAN, B. M. 2012. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell,* 150**,** 366-76.

WU, Z., ROSEN, E. D., BRUN, R., HAUSER, S., ADELMANT, G., TROY, A. E., MCKEON, C., DARLINGTON, G. J. & SPIEGELMAN, B. M. 1999. Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol Cell,* 3**,** 151-8.

WWF. 2013. *Impact of habitat loss on species* [Online]. Gland, Switzerland: WWF. Available: http://wwf.panda.org/about_our_earth/species/problems/habitat_loss_degradation/ [Accessed 14 August 2013 2013].

WWF. 2015. *African elephants* [Online]. Gland, Switzerland: World Wide Fund for Nature (WWF). [Accessed 16 July 2015 2015].
WYLIE, D. 2008. Using elephants. *Elephants.* London, UK: Reaktion Books Ltd.
XIE, B., HUANG, R., HUANG, L., ZHOU, G. & GONG, Z. 2003. The functional domains of human ventricular myosin light chain 1. *Biophys Chem,* 106**,** 57-66.
XIE, R., XU, J., WEN, G., JIN, H., LIU, X., YANG, Y., JI, B., JIANG, Y., SONG, P., DONG, H. & TUO, B. 2014. The P2Y2 nucleotide receptor mediates the proliferation and migration of human hepatocellular carcinoma cells induced by ATP. *J Biol Chem,* 289**,** 19137-49.
YAMAKAWA, T., SUGIMOTO, K., WHITSON, R. H. & ITAKURA, K. 2010. Modulator recognition factor-2 regulates triglyceride metabolism in adipocytes. *Biochem Biophys Res Commun,* 391**,** 277-81.
YAMAKAWA, T., WHITSON, R. H., LI, S. L. & ITAKURA, K. 2008. Modulator recognition factor-2 is required for adipogenesis in mouse embryo fibroblasts and 3T3-L1 cells. *Mol Endocrinol,* 22**,** 441-53.
YAMAUCHI, T., WAKI, H., KAMON, J., MURAKAMI, K., MOTOJIMA, K., KOMEDA, K., MIKI, H., KUBOTA, N., TERAUCHI, Y., TSUCHIDA, A., TSUBOYAMA-KASAOKA, N., YAMAUCHI, N., IDE, T., HORI, W., KATO, S., FUKAYAMA, M., AKANUMA, Y., EZAKI, O., ITAI, A., NAGAI, R., KIMURA, S., TOBE, K., KAGECHIKA, H., SHUDO, K. & KADOWAKI, T. 2001. Inhibition of RXR and PPARγ ameliorates diet-induced obesity and type 2 diabetes. *Journal of Clinical Investigation,* 108**,** 1001-1013.
YANG, R. Y., HSU, D. K., YU, L., CHEN, H. Y. & LIU, F. T. 2004. Galectin-12 is required for adipogenic signaling and adipocyte differentiation. *J Biol Chem,* 279**,** 29761-6.
YANG, R. Y., YU, L., GRAHAM, J. L., HSU, D. K., LLOYD, K. C., HAVEL, P. J. & LIU, F. T. 2011. Ablation of a galectin preferentially expressed in adipocytes increases lipolysis, reduces adiposity, and improves insulin sensitivity in mice. *Proc Natl Acad Sci U S A,* 108**,** 18696-701.
YEE, J. K., WAHJUDI, P. N., VEGA, J., LIM, S., MARTIN, A., PATTERSON, M. E., COHEN, J. N., MAO, C. S. & LEE, W.-N. P. 2013. Stearoyl-CoA desaturase enzyme 1 inhibition reduces glucose utilization for de novo fatty acid synthesis and cell proliferation in 3T3-L1 adipocytes. *Metabolomics : Official journal of the Metabolomic Society,* 9**,** 809-816.
YEH, W. C., CAO, Z., CLASSON, M. & MCKNIGHT, S. L. 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev,* 9**,** 168-81.
YOKOYAMA, C., WANG, X., BRIGGS, M. R., ADMON, A., WU, J., HUA, X., GOLDSTEIN, J. L. & BROWN, M. S. 1993. SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell,* 75**,** 187-197.
YOUNG, P., ARCH, J. R. & ASHWELL, M. 1984. Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Lett,* 167**,** 10-4.
ZAFRA, M. P., CANAS, J. A., MAZZEO, C., GAMEZ, C., SANZ, V., FERNANDEZ-NIETO, M., QUIRCE, S., BARRANCO, P., RUIZ-HORNILLOS, J., SASTRE, J. & DEL POZO, V. 2015. SOCS3 silencing attenuates eosinophil functions in asthma patients. *Int J Mol Sci,* 16**,** 5434-51.
ZAIDI, N., LUPIEN, L., KUEMMERLE, N. B., KINLAW, W. B., SWINNEN, J. V. & SMANS, K. 2013. Lipogenesis and lipolysis: The pathways exploited by the cancer cells to acquire fatty acids. *Progress in Lipid Research,* 52**,** 585-589.
ZEC, N., ROWITCH, D. H., BITGOOD, M. J. & KINNEY, H. C. 1997. Expression of the homeobox-containing genes EN1 and EN2 in human fetal midgestational medulla and cerebellum. *J Neuropathol Exp Neurol,* 56**,** 236-42.

ZEMEL, M. B. 2003. Mechanisms of Dairy Modulation of Adiposity. *The Journal of Nutrition,* 133**,** 252S-256S.

ZHANG, H., JIA, Y., COOPER, J. J., HALE, T., ZHANG, Z. & ELBEIN, S. C. 2004. Common variants in glutamine:fructose-6-phosphate amidotransferase 2 (GFPT2) gene are associated with type 2 diabetes, diabetic nephropathy, and increased GFPT2 mRNA levels. *J Clin Endocrinol Metab,* 89**,** 748-55.

ZHANG, J., XU, E., REN, C., YAN, W., ZHANG, M., CHEN, M., CARDIFF, R. D., IMAI, D. M., WISNER, E. & CHEN, X. 2014a. Mice deficient in Rbm38, a target of the p53 family, are susceptible to accelerated aging and spontaneous tumors. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 18637-18642.

ZHANG, Q., LEE, H. G., HAN, J. A., KIM, E. B., KANG, S. K., YIN, J., BAIK, M., SHEN, Y., KIM, S. H., SEO, K. S. & CHOI, Y. J. 2010. Differentially expressed proteins during fat accumulation in bovine skeletal muscle. *Meat Sci,* 86**,** 814-20.

ZHANG, Q., WIDMER, G. & TZIPORI, S. 2013. A pig model of the human gastrointestinal tract. *Gut Microbes,* 4**,** 193-200.

ZHANG, Y., HUANG, H., HUANG, J., XIANG, Z., YANG, M., TIAN, C. & FAN, H. 2012. The -444A/C polymorphism in the LTC4S gene and the risk of asthma: a meta-analysis. *Arch Med Res,* 43**,** 444-50.

ZHANG, Z. H., JHAVERI, D. J., MARSHALL, V. M., BAUER, D. C., EDSON, J., NARAYANAN, R. K., ROBINSON, G. J., LUNDBERG, A. E., BARTLETT, P. F., WRAY, N. R. & ZHAO, Q. Y. 2014b. A Comparative Study of Techniques for Differential Expression Analysis on RNA-Seq Data. *PLoS One,* 9**,** e103207.

ZHUANG, H., LIN, Y. & YANG, G. 2007. Effects of 1,25-dihydroxyvitamin D3 on proliferation and differentiation of porcine preadipocyte in vitro. *Chem Biol Interact,* 170**,** 114-23.

ZIMMERMANN, R., LASS, A., HAEMMERLE, G. & ZECHNER, R. 2009. Fate of fat: The role of adipose triglyceride lipase in lipolysis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1791**,** 494-500.

ZIZOLA, C. F., SCHWARTZ, G. J. & VOGEL, S. 2008. Cellular retinol-binding protein type III is a PPARgamma target gene and plays a role in lipid metabolism. *Am J Physiol Endocrinol Metab,* 295**,** E1358-68.

ZUKOSKY, K., MEILLEUR, K., TRAYNOR, B. J., DASTGIR, J., MEDNE, L., DEVOTO, M., COLLINS, J., ROONEY, J., ZOU, Y., YANG, M. L., GIBBS, J. R., MEIER, M., STETEFELD, J., FINKEL, R. S., SCHESSL, J., ELMAN, L., FELICE, K., FERGUSON, T. A., CEYHAN-BIRSOY, O., BEGGS, A. H., TENNEKOON, G., JOHNSON, J. O. & BONNEMANN, C. G. 2015. Association of a Novel ACTA1 Mutation With a Dominant Progressive Scapuloperoneal Myopathy in an Extended Family. *JAMA Neurol,* 72**,** 689-98.

Appendix 1

1. QC and trimming using Cutadapt, Sickle and FastQC:

/home/ADAC_guest/tools/scripts/fastq.cutadapt.sickle.fastqc.pipeline.pl -i fastq.cutadapt.sickle.fastqc.pipeline.pl.test.control -f fastqc -s sickle -e sanger -q 20 &

a) fastq.cutadapt.sickle.fastqc.pipeline.pl:

```
#!/usr/bin/perl
use strict;
use warnings;
# batch process a directory of fastq files , cut adapt, sickle, fastqc

use Getopt::Long;

#
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~

# Richard Emes University of Nottingham 2013

my $usage = "

@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@
@@@@@@@@@@@@@@@@@@

Richard Emes University of Nottingham 2013


batch process a directory of fastq files with

1) cut adapt

2) sickle

3) fastqc


ASSUMES cutadapt is in your path.

Read files can be zipped (assumed ends in .gz)

USAGE:

-i	input control file (tab delimited no headers [\"PE\" or \"SE\" paired or single] [file 1] [file 2 (if PE)] [Adapter to trim file 1] [Adapter to trim file 2 (if PE)])

-f	path to fastqc (~/tools/FastQC/fastqc)

-s	path to sickle (/home/rde/tools/Sickle/najoshi-sickle-032bf41/sickle)

-e	illumina encoding type [sanger, solexa or illumina RUN ~/tools/perl/scripts/NGS_Sequencing/DetermineFastqQualityEncoding.pl to determine]

-q	trimming quality e.g 20, 30 for sickle average quality in a window
```

```
@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@@
@@@@@@@@@@@@@@@@@@

" ;

my ($input, $fastqc, $sickle, $encoding, $quality);


GetOptions(

	'i|input:s'	=> \$input,

	'f|fastqc:s'	=> \$fastqc,

	's|sickle:s'	=> \$sickle,

	'e|encoding:s'	=> \$encoding,

	'q|quality:s'	=> \$quality,

	);


if( ! defined $input) {

print "\n$usage\nWARNING: Cannot proceed without input file\n\n"; exit;

}

if( ! defined $fastqc) {

print "\n$usage\nWARNING: Cannot proceed without path to FastQC\n\n"; exit;

}

if( ! defined $sickle) {

print "\n$usage\nWARNING: Cannot proceed without path to Sickle\n\n"; exit;

}

if( ! defined $encoding) {

print "\n$usage\nWARNING: Cannot proceed without sequence encoding type\n\n"; exit;

}

if( ! defined $quality) {

print "\n$usage\nWARNING: Cannot proceed without quailty cutoff for sickle\n\n"; exit;

}


open CONTROL, "$input";
```

```
my $line = 1;

my $error_flag = 0;

##### CHECK INPUT BEFORE RUNNING
#####################################################################

print "\n-----------------------------------------------------\nCHECKING INPUT\n";

while (<CONTROL>)

{

my @data = split '\t', $_;

my $end = $data[0];


if ($end eq "PE" || $end eq "pe") {if (scalar @data != 5){print "ERROR\: Missing infomation line
$line of $input\n";$error_flag = 1}}

if ($end eq "SE" || $end eq "se") {if (scalar @data != 3){print "ERROR\: Missing infomation line
$line of $input\n";$error_flag = 1}}

$line++;

}

close CONTROL;

if ($error_flag == 1) {print "CORRECT ERRORS in $input BEFORE RERUNNING\n----------------
-----------------------------------\n";exit;}
#############################################################################################
#######

my $count = 1;

if ($error_flag == 0) {print "FILES APPEAR OK\n-----------------------------------------------------\n";

        open LOG, ">$input\.QC\.log";

        open  CONTROL, "$input";

        while (<CONTROL>)

        {

        print "Processing File $count\n";
        my @data = split '\t', $_;

        my $end = $data[0];

        if ($end eq "PE" || $end eq "pe")
                {

                my $file1 = $data[1];
```

```
my $file2 = $data[2];

my $directory1 = "./";

my $directory2 = "./";

if ($file1 =~ /(.*\/)(.*)/){$directory1 = $1;$file1 = $2;}

if ($file2 =~ /(.*\/)(.*)/){$directory2 = $1;$file2 = $2;}


if ($directory1 =~ /(.*?)\/$/){$directory1 = $1;}

if ($directory2 =~ /(.*?)\/$/){$directory2 = $1;}


my $adapter1 = $data[3];

my $adapter2 = $data[4];


my $name1 = $file1;

my $name2 = $file2;

chomp ($file1,$file2,$adapter1,$adapter2,$name1,$name2,$directory1,$directory2);

my $adapter1_RC = reverse $adapter1;

$adapter1_RC =~ tr/ACGTacgt/TGCAtgca/;


my $adapter2_RC = reverse $adapter2;

$adapter2_RC =~ tr/ACGTacgt/TGCAtgca/;


print LOG "\n----------\n$name1\t$name2\n";


if ($file1 =~ /((.*)\.gz$)/)

{

system "gunzip $file1";

$name1 = $2;

}

if ($file2 =~ /((.*)\.gz$)/)

{

system "gunzip $file2";
```

```
		$name2 = $2;

		}

		system "cutadapt -b $adapter1 \ -b $adapter1_RC -e 0.1 -O 5 -o
$directory1\/$name1.trimmed.fastq $directory1\/$name1 >> $input\.QC\.log";

		system "cutadapt -b $adapter2 \ -b $adapter2_RC -e 0.1 -O 5 -o
$directory2\/$name2.trimmed.fastq $directory2\/$name2 >> $input\.QC\.log";

		print  LOG "SICKLE commands\n$sickle pe -f $directory1\/$name1.trimmed.fastq
-r $directory2\/$name2.trimmed.fastq -t $encoding -q $quality -o
$directory1\/$name1.trimmed.QC.fastq -p $directory2\/$name2.trimmed.QC.fastq -s
$directory1\/$name1\_$name2\_singles.trimmed.QC.fastq\n";

		system "gzip -c $directory1\/$name1 > $directory1\/$name1\.gz";

		system "gzip -c $directory1\/$name2 > $directory2\/$name1\.gz";


		system "rm $directory1\/$name1";

		system "rm $directory1\/$name2";


		system "$sickle pe -f $directory1\/$name1.trimmed.fastq -r
$directory2\/$name2.trimmed.fastq -t $encoding -q $quality -o
$directory1\/$name1.trimmed.QC.fastq -p $directory2\/$name2.trimmed.QC.fastq -s
$directory1\/$name1\_$name2\_singles.trimmed.QC.fastq >> $input\.QC\.log";

		print  LOG "FASTQC commands\n$fastqc -quiet
	$directory1\/$name1.trimmed.QC.fastq\n";

		print  LOG "$fastqc --quiet $directory2\/$name2.trimmed.QC.fastq\n";

		system "$fastqc --quiet $directory1\/$name1.trimmed.QC.fastq";

		system "$fastqc --quiet $directory2\/$name2.trimmed.QC.fastq";


		system "rm $directory1\/$name1.trimmed.fastq";

		system "rm $directory1\/$name2.trimmed.fastq";


		if (-e "$directory1\/FastQC_reports"){}

		else {system "mkdir $directory1\/FastQC_reports";}


		if (-e "$directory1\/Cutadapt.plus.SickleQC.trimmed"){}

		else {system "mkdir $directory1\/Cutadapt.plus.SickleQC.trimmed";}


if (-e "$directory2\/FastQC_reports"){}
```

```
		else {system "mkdir $directory2\/FastQC_reports";}

		if (-e "$directory2\/Cutadapt.plus.SickleQC.trimmed"){}

		else {system "mkdir $directory2\/Cutadapt.plus.SickleQC.trimmed";}

		system "mv $directory1\/*\_fastqc $directory1\/FastQC_reports/";

		system "rm $directory1\/*\_fastqc.zip";

		system "mv $directory1\/*\.trimmed.QC.fastq
$directory1/Cutadapt.plus.SickleQC.trimmed/";

		if (-e "$directory2\/*\_fastqc") {system "mv $directory2\/*\_fastqc
$directory2\/FastQC_reports/";}

		if (-e "$directory2\/*\_fastqc.zip") {system "rm $directory2\/*\_fastqc.zip";}

		if (-e "$directory2\/*\.trimmed.QC.fastq") {system "mv
$directory2\/*\.trimmed.QC.fastq $directory2\/Cutadapt.plus.SickleQC.trimmed/";}

		}

		if ($end eq "SE" || $end eq "se")

		{

		my $file1 = $data[1];

		my $directory1 = "./";

		if ($file1 =~ /(.*\/)(.*)/){$directory1 = $1;$file1 = $2;}

		if ($directory1 =~ /(.*?)\/$/){$directory1 = $1;}

		my $adapter1 = $data[2];

		my $name1 = $file1;

		chomp ($file1,$adapter1,$name1,$directory1);

		my $adapter1_RC = reverse $adapter1;

		$adapter1_RC =~ tr/ACGTacgt/TGCAtgca/;

		print LOG "\n-----------------------------------------------------\n$name1\n";

		if ($file1 =~ /((.*)\.gz$)/)

		{
```

```
                system "gunzip $file1";

                $name1 = $2;
                }

                system "cutadapt -a $adapter1 \ -a $adapter1_RC -e 0.1 -O 5 -o
$directory1\/$name1.trimmed.fastq $directory1\/$name1 >> $input\.QC\.log";

                system "gzip -c $directory1\/$name1 > $directory1\/$name1\.gz";

                system "rm $directory1\/$name1";


                print  LOG "SICKLE commands\n$sickle se -f $directory1\/$name1.trimmed.fastq
-t $encoding -q $quality -o $directory1\/$name1.trimmed.QC.fastq\n";

                system "$sickle se -f $directory1\/$name1.trimmed.fastq -t $encoding -q $quality -
o $directory1\/$name1.trimmed.QC.fastq >> $input\.QC\.log";

                print  LOG "FASTQC commands\n$fastqc -quiet
$directory1\/$name1.trimmed.QC.fastq\n";


                system "$fastqc --quiet $directory1\/$name1.trimmed.QC.fastq";


                system "rm $directory1\/$name1.trimmed.fastq";


                if (-e "$directory1\/FastQC_reports"){}

                else {system "mkdir $directory1\/FastQC_reports";}


                if (-e "$directory1\/Cutadapt.plus.SickleQC.trimmed"){}

                else {system "mkdir $directory1\/Cutadapt.plus.SickleQC.trimmed";}


                system "mv $directory1\/*\_fastqc $directory1\/FastQC_reports/";

                system "rm $directory1\/*\_fastqc.zip";

                system "mv $directory1\/*\.trimmed.QC.fastq
$directory1/Cutadapt.plus.SickleQC.trimmed/";

                }


                  $count++
        }

}
```

b) fastq.cutadapt.sickle.fastqc.pipeline.pl.test.control:

```
PE      /home/ADAC_guest/Sandie.expression.data/sequence.reads/Cow/0833N0009_ATCACG_L007_R1_001.fastq
        /home/ADAC_guest/Sandie.expression.data/sequence.reads/Cow/0833N0009_ATCACG_L007_R2_001.fastq
        ACACGACGCTCTTCCGATCT        ACACGACGCTCTTCCGATCT
PE      /home/ADAC_guest/Sandie.expression.data/sequence.reads/Cow/0833N0009_ATCACG_L008_R1_001.fastq
        /home/ADAC_guest/Sandie.expression.data/sequence.reads/Cow/0833N0009_ATCACG_L008_R2_001.fastq
        ACACGACGCTCTTCCGATCT        ACACGACGCTCTTCCGATCT
```

2. Create reference genome index file:

bowtie2-build –q Bos_taurus.UMD3.1.71.dna.toplevel.fa Bos_taurus

3. Mapping to reference genome using TopHat:

tophat -r 50 -p 4 -G /home/ADAC_guest/Sandie.expression.data/Genomes/
Cow/Bos_taurus.UMD3.1.71.gtf -o tophat_0833N0009
/home/ADAC_guest/Sandie.expression.data/Genomes/Cow/Bos_taurus
0833N0009_ATCACG_L007_R1_001.fastq.trimmed.QC.fastq,0833N0009_ATCACG_L008_
R1_001.fastq.trimmed.QC.fastq
0833N0009_ATCACG_L007_R2_001.fastq.trimmed.QC.fastq,0833N0009_ATCACG_L008_
R2_001.fastq.trimmed.QC.fastq

4. Annotate to reference genome using Cufflink:

cufflinks -o
/home/ADAC_guest/Sandie.expression.data/sequence.reads/Project833_adipose/Raw.read
s.and.QC/Cow/Cutadapt.plus.SickleQC.trimmed/cufflinks_0833N0009 -p 4 -G
/home/ADAC_guest/Sandie.expression.data/Genomes/Cow/Bos_taurus.UMD3.1.71.gtf
/home/ADAC_guest/Sandie.expression.data/sequence.reads/Project833_adipose/Raw.read
s.and.QC/Cow/Cutadapt.plus.SickleQC.trimmed/tophat_0833N0009/accepted_hits.bam

5. Convert bam files into HTSEQ count files:

samtools sort -n
/home/ADAC_guest/Sandie.expression.data/sequence.reads/top.hat.bamfiles/Cow/0833N0009.b
am
/home/ADAC_guest/Sandie.expression.data/sequence.reads/top.hat.bamfiles/Cow/0833N0009.b
am.sorted

rm
/home/ADAC_guest/Sandie.expression.data/sequence.reads/top.hat.bamfiles/Cow/0833N0009.b
am

htseq-count -f bam --mode=union
/home/ADAC_guest/Sandie.expression.data/sequence.reads/top.hat.bamfiles/Cow/0833N0009.b
am.sorted.bam
/home/ADAC_guest/Sandie.expression.data/Genomes/Cow/Bos_taurus.UMD3.1.71.gtf >
/home/ADAC_guest/Sandie.expression.data/sequence.reads/HTSEQ.count.files/Cow/0833N000
9.counts

6. Combine HTSEQ files of samples for DGE:

paste
/home/ADAC_guest/Sandie.expression.data/sequence.reads/HTSEQ.count.files/Cow/0833N0009.counts
/home/ADAC_guest/Sandie.expression.data/sequence.reads/HTSEQ.count.files/Cow/0833N0010.counts /home/ADAC_guest/Sandie.expression.data/sequence
reads/HTSEQ.count.files/Cow/1160N0002.counts
/home/ADAC_guest/Sandie.expression.data/sequence.reads/HTSEQ.count.files/Cow1160N0003.counts > a

7. Remove other columns of gene Ensembl identifiers except the first, and rename the file:

cut –f1,2,4,6,8 a>Holstein.vs.Hereford.counts

8. Add header to the top of the list:

sed gene iGt 1.1\1.2\2.1\2.2

9. Remove "a file" created in the combination step above:

rm a

10. DGE analysis using edgeR:

R --vanilla < run.EdgeR.Holstein.vs.Hereford.R

a) run.EdgeR.Holstein.vs.Hereford.R:

```
#source("http://bioconductor.org/biocLite.R")
#biocLite("edgeR")
library(edgeR)
library(limma)

################################################################################
##############################
## USER TO CHANGE
setwd('/home/ADAC_guest/Sandie.expression.data/sequence.reads/EdgeR.analysis/Cow')

x <- read.delim("Holstein.vs.Hereford.counts",row.names="GENE")

group <- factor(c(1,1,2))

all_results_table = "Holstein.vs.Hereford_allResults_edgeR.xls"
```

```
de_ids_table = "Holstein.vs.Hereford_DE-IDs_edgeR.xls"
FDR.lt0.05_table = "Holstein.vs.Hereford_DE_edgeR.xls"
FDR.lt0.05_FC2.table = "Holstein.vs.Hereford_DE_FC2.edgeR.xls"

mds.plot.name = "Holstein.vs.Hereford.MDS.plot.pdf"
smear.plot.name = "Holstein.vs.Hereford.smear.plot.pdf"
volcano.plot.name = "Holstein.vs.Hereford.volcano.plot.pdf"

################################################################################
##############################

y <- DGEList(counts=x,group=group)

## Since the smallest group size is TWO, we keep genes that achieve at least one count per
million (cpm) in at least TWO samples:\n";
keep <- rowSums(cpm(y)>1) >= 2
y <- y[keep,]
# Re-compute the library sizes
y$samples$lib.size <- colSums(y$counts)

# Compute effective library sizes using TMM normalization:\n";
y <- calcNormFactors(y)
y$samples

#  Output plot as a pdf
pdf(mds.plot.name)
plotMDS(y)
dev.off()


y <- estimateCommonDisp(y)
y <- estimateTagwiseDisp(y)
#plotBCV(y)
et <- exactTest(y)
top <- topTags(et)

pdf(smear.plot.name)
plotSmear(y, de.tags=top, main="Differential expression using the common dispersion")
dev.off()

## Check the individual cpm values for the top genes:
cpm(y)[rownames(top), ]
## The total number of DE genes at 5% FDR is given by:
summary(de <- decideTestsDGE(et))
## Plot the log-fold-changes, highlighting the DE genes:
detags <- rownames(y)[as.logical(de)]

# volcano plot
pdf(volcano.plot.name)
plot(et$table$logFC, -log10(et$table$PValue), pch=19, cex=0.2, main  ="Volcano Plot", xlab =
"LogFC", ylab = "-log10 pvalue")
dev.off()

allResults <- topTags( et , n = nrow( et$table ) )$table
gene.counts <- y$counts
combined.results <- merge(allResults, gene.counts, by=0, all=TRUE)
combined.results.UP.G2 <- subset(combined.results, logFC > 0)
```

```
combined.results.DOWN.G2 <- subset(combined.results, logFC <= 0)

combined.results.UP.G2[, "G2vsG1"] <- "UP"
combined.results.DOWN.G2[, "G2vsG1"] <- "DOWN"

combined.results <- rbind(combined.results.UP.G2,combined.results.DOWN.G2)

significant.results <- subset(combined.results, FDR <= 0.05)
significant.results.FC2.UP.G2 <- subset(significant.results, logFC >= 1)
significant.results.FC2.DOWN.G2 <- subset(significant.results, logFC <= -1)

combined.significant.FC2 <-
rbind(significant.results.FC2.UP.G2,significant.results.FC2.DOWN.G2)


write.table(combined.results,
file=all_results_table,row.names=FALSE,col.names=TRUE,quote=FALSE)
write.table(significant.results,
file=FDR.lt0.05_table,row.names=FALSE,col.names=TRUE,quote=FALSE)
write.table(detags, file=de_ids_table,row.names=FALSE,col.names=FALSE,quote=FALSE)
write.table(combined.significant.FC2,
file=FDR.lt0.05_FC2.table,row.names=FALSE,col.names=TRUE,quote=FALSE)
```

Appendix 2

Top 100 up and downregulated DEG in AT of Holstein versus Hereford (FC ≥ 2, FDR < 0.05)

Ensembl ID	Gene symbol	logFC
ENSBTAG00000003989		5.771333
ENSBTAG00000017040	LY6E	4.668573
ENSBTAG00000002786	LOC781494	4.222637
ENSBTAG00000009570	C19orf80	3.987404
ENSBTAG00000034077	ASIP	3.914065
ENSBTAG00000015441	ACTB	3.844798
ENSBTAG00000000575	TNC	3.843350
ENSBTAG00000047764		3.692456
ENSBTAG00000016740	ACLY	3.537042
ENSBTAG00000015988	MYH11	3.522442
ENSBTAG00000007237	BUB1B	3.505223
ENSBTAG00000006882	IQGAP3	3.498849
ENSBTAG00000009819	CDC20	3.350713
ENSBTAG00000030483	KLK7	3.337822
ENSBTAG00000001592	INSIG1	3.316031
ENSBTAG00000005353	DES	3.311461
ENSBTAG00000001785	TGM3	3.263808
ENSBTAG00000031020		3.260531
ENSBTAG00000025181	CCDC85C	3.247082
ENSBTAG00000008105	RBM38	3.220405
ENSBTAG00000000590	POLE	3.197266
ENSBTAG00000014088	MBNL3	3.187393
ENSBTAG00000043683		3.157202
ENSBTAG00000002444	MKI67	3.153828
ENSBTAG00000012640	S100A8	3.137138
ENSBTAG00000005260	SPP1	3.122980
ENSBTAG00000021494	EN1	3.092303
ENSBTAG00000011780	SLITRK4	3.044180
ENSBTAG00000002940	WNT16	3.011044
ENSBTAG00000015919	DKFZP564O0823	3.005690
ENSBTAG00000047957	SCD	3.002788
ENSBTAG00000007169	P2RX1	2.973553
ENSBTAG00000006505	S100A9	2.903486
ENSBTAG00000046753		2.872080
ENSBTAG00000019262	TOP2A	2.864704
ENSBTAG00000014614	ACTA2	2.836588
ENSBTAG00000038700	FAM124B	2.818832
ENSBTAG00000032059	TGM5	2.818451
ENSBTAG00000013347	DMPK	2.803564
ENSBTAG00000045728	SCD	2.789424
ENSBTAG00000017567	ACACA	2.787692
ENSBTAG00000014575	COL4A5	2.747651
ENSBTAG00000004510	SARDH	2.732708
ENSBTAG00000019293	EGR3	2.708889
ENSBTAG00000008216	RRM2	2.678369
ENSBTAG00000015160	PLD4	2.673424
ENSBTAG00000038124	FCGR2C	2.650460
ENSBTAG00000044208	DUSP4	2.620014
ENSBTAG00000034435	NKD2	2.589295
ENSBTAG00000001462	FLT4	2.571662
ENSBTAG00000012057	GIF	-8.742030
ENSBTAG00000019870	SLC14A1	-7.031310
ENSBTAG00000005305	NTS	-5.373340
ENSBTAG00000027348	OOSP1	-5.356230
ENSBTAG00000007239	TSG-6	-5.258930
ENSBTAG00000037605	BOLA-DQA1	-5.163550
ENSBTAG00000002929	IRF4	-4.623520
ENSBTAG00000016399	AKAP4	-4.433700
ENSBTAG00000031532	DACT2	-4.065030
ENSBTAG00000001392	RDH16	-4.032950
ENSBTAG00000000118		-4.01500
ENSBTAG00000003957		-3.98831
ENSBTAG00000046420	SNORD113	-3.96084

ENSBTAG00000038461		-3.89909
ENSBTAG00000009870	HES6	-3.80481
ENSBTAG00000015374	COL22A1	-3.51475
ENSBTAG00000003408		-3.45056
ENSBTAG00000018540	NOX4	-3.43827
ENSBTAG00000007241	SLAMF9	-3.36955
ENSBTAG00000017233		-3.30974
ENSBTAG00000001862	PPP2R2B	-3.21030
ENSBTAG00000017366	GRB7	-3.18885
ENSBTAG00000033803	FABP7	-3.17693
ENSBTAG00000012071	ASGR1	-3.10848
ENSBTAG00000004662	SLC16A12	-3.09386
ENSBTAG00000043378	SNORD81	-2.98098
ENSBTAG00000004355	ROBO3	-2.93394
ENSBTAG00000018571	IL1RL1	-2.93354
ENSBTAG00000037452		-2.93247
ENSBTAG00000011002	CCDC136	-2.92651
ENSBTAG00000047547		-2.91636
ENSBTAG00000002215	GFPT2	-2.89505
ENSBTAG00000025642	RYR3	-2.86489
ENSBTAG00000019892	HAS2	-2.84917
ENSBTAG00000001388	NMB	-2.83942
ENSBTAG00000014407	INO80D	-2.78803
ENSBTAG00000013213	OSR2	-2.77744
ENSBTAG00000000277	IL18	-2.77144
ENSBTAG00000011976	CYP4B1	-2.75425
ENSBTAG00000015821	AMPD3	-2.63397
ENSBTAG00000016234	DUOX2	-2.61403
ENSBTAG00000032591	CTHRC1	-2.57709
ENSBTAG00000021672	RGS1	-2.57233
ENSBTAG00000039728	RPLP1	-2.55655
ENSBTAG00000012357		-2.53533
ENSBTAG00000002670	C28H10ORF10	-2.51839
ENSBTAG00000017442	CDO1	-2.51004
ENSBTAG00000005910	LEPR	-2.50237
ENSBTAG00000043468	SNORD50	-2.50237
ENSBTAG00000019822	TPPP3	-2.49446

Appendix 3

Top 100 up and downregulated DEG in AT of LP versus HP (FC ≥ 2, FDR < 0.05)

Ensembl ID	Gene symbol	logFC
ENSBTAG00000003212	NNAT	3.71574
ENSBTAG00000045928		3.55696
ENSBTAG00000005910	LEPR	3.46382
ENSBTAG00000007846	ITIH3	2.62749
ENSBTAG00000012715	KIF26B	2.59606
ENSBTAG00000017369	MAMDC2	2.39055
ENSBTAG00000047147		2.27400
ENSBTAG00000000859	SLC38A1	1.96327
ENSBTAG00000016768	SCN3B	1.93776
ENSBTAG00000045702	ZBED6	1.91936
ENSBTAG00000015214	CA3	1.84383
ENSBTAG00000031802	SPATA16	1.81072
ENSBTAG00000011088	SLC6A6	1.73046
ENSBTAG00000038043	ABCD2	1.68972
ENSBTAG00000004443		1.68303
ENSBTAG00000004901	PIK3C2A	1.66074
ENSBTAG00000001879	PER2	1.64991
ENSBTAG00000044208	DUSP4	1.64518
ENSBTAG00000002996	SHROOM4	1.64225
ENSBTAG00000011932	PRG4	1.63503
ENSBTAG00000005527	GIPR	1.61867
ENSBTAG00000020520	RASD1	1.59728
ENSBTAG00000039050	P2RY2	1.57189
ENSBTAG00000007843	ITIH1	1.55726
ENSBTAG00000045685		1.54973
ENSBTAG00000013791	GPR41	1.53872
ENSBTAG00000010018	ATP7A	1.51965
ENSBTAG00000034985	PHLDA1	1.51190
ENSBTAG00000009691	SH2B2	1.45732
ENSBTAG00000033429	FAM229B	1.42279
ENSBTAG00000002979	PIK3R3	1.42241
ENSBTAG00000047766	G0S2	1.41847
ENSBTAG00000012866	THBS4	1.41261
ENSBTAG00000020247	ADCYAP1R1	1.38898
ENSBTAG00000007946	PIPOX	1.37493
ENSBTAG00000023289	SLC26A10	1.34153
ENSBTAG00000017969	CA4	1.31929
ENSBTAG00000010366	HCRTR1	1.31762
ENSBTAG00000008493	AQP3	1.31509
ENSBTAG00000020747	ASS1	1.30817
ENSBTAG00000007694	KIF25	1.29765
ENSBTAG00000010423	LIFR	1.27714
ENSBTAG00000010244	CLIC5	1.26397
ENSBTAG00000047621		1.26162
ENSBTAG00000007884	SREBF1	1.24509
ENSBTAG00000005419	ABP1	1.23558
ENSBTAG00000005556	CYGB	1.23090
ENSBTAG00000008250	SPRY4	1.22357
ENSBTAG00000005370	TMTC1	1.21777
ENSBTAG00000023947	AVPI1	1.20252
ENSBTAG00000021310	COL4A4	-4.21481
ENSBTAG00000038584	OLFM1	-4.16615
ENSBTAG00000034498	LY6D	-3.44515
ENSBTAG00000034662		-3.33855
ENSBTAG00000004647	UCP1	-3.01268
ENSBTAG00000036343	LOC526488	-2.82371
ENSBTAG00000000828	CAPN6	-2.74837
ENSBTAG00000017007	TRIB3	-2.73271
ENSBTAG00000003367		-2.52643
ENSBTAG00000012991	PRUNE2	-2.47261
ENSBTAG00000009570	C19orf80	-2.43596
ENSBTAG00000015080	PHACTR3	-2.32235
ENSBTAG00000002075	MME	-2.29908

ENSBTAG00000030322	KLHDC7A	-2.27532
ENSBTAG00000009174	HSD17B14	-2.23870
ENSBTAG00000009831	OCA2	-2.20338
ENSBTAG00000038058	LOC518623	-2.15803
ENSBTAG00000001021	CYP1A1	-2.15480
ENSBTAG00000011076	KCNN2	-2.10773
ENSBTAG00000000284	NAALAD2	-2.05034
ENSBTAG00000045728	SCD	-2.03470
ENSBTAG00000019807	COL27A1	-2.02353
ENSBTAG00000047957	SCD	-1.99466
ENSBTAG00000015980	FASN	-1.98932
ENSBTAG00000021033	SGK2	-1.90196
ENSBTAG00000006991	ADH6	-1.83545
ENSBTAG00000006349	EXTL1	-1.80828
ENSBTAG00000024647		-1.80109
ENSBTAG00000004510	SARDH	-1.78262
ENSBTAG00000000448	BDH1	-1.77931
ENSBTAG00000000590	POLE	-1.76187
ENSBTAG00000002138	PADI1	-1.69212
ENSBTAG00000025258		-1.68235
ENSBTAG00000018517	VLDLR	-1.67588
ENSBTAG00000037899	DLK	-1.67348
ENSBTAG00000008528	SLC25A1	-1.66655
ENSBTAG00000020050	MLEC	-1.66315
ENSBTAG00000025181	CCDC85C	-1.65201
ENSBTAG00000039708	C9orf172	-1.63755
ENSBTAG00000015919	DKFZP564O0823	-1.63551
ENSBTAG00000002299	SEL1L3	-1.62301
ENSBTAG00000032059	TGM5	-1.60800
ENSBTAG00000007111	STEAP3	-1.60341
ENSBTAG00000016740	ACLY	-1.56662
ENSBTAG00000031184	CDKN1C	-1.56254
ENSBTAG00000037539	VCAM1	-1.55061
ENSBTAG00000024379	LRRC17	-1.54751
ENSBTAG00000039520	SIRPB1	-1.52658
ENSBTAG00000025071	TENM2	-1.52635
ENSBTAG00000013303	ACSS2	-1.52379

Appendix 4

Top 100 up and downregulated DEG in AT of MS versus LW (FC ≥ 2, FDR < 0.05)

Ensembl ID	Gene symbol	logFC
ENSSSCG00000016516	ATP6V0A4	6.61525
ENSSSCG00000011325	MYL3	6.27025
ENSSSCG00000010007	MTFP1	6.25987
ENSSSCG00000024061	TNNI1	6.12909
ENSSSCG00000025353	TNNT1	6.07180
ENSSSCG00000002029	MYH7	5.83333
ENSSSCG00000011441	TNNC1	5.63247
ENSSSCG00000009830	MLC-2V	5.61053
ENSSSCG00000030999	MYH6	5.42214
ENSSSCG00000013354	CSRP3	5.26592
ENSSSCG00000010461	ANKRD1	5.11966
ENSSSCG00000024422		4.92169
ENSSSCG00000007505	CTCFL	4.88115
ENSSSCG00000026162		4.79266
ENSSSCG00000018007	MYH3	4.52867
ENSSSCG00000010814	ESRRG	4.51974
ENSSSCG00000021071	HSPB7	4.47810
ENSSSCG00000022737	MYD88	4.38989
ENSSSCG00000021375	HS6ST2	4.37937
ENSSSCG00000004803	ACTC1	4.28798
ENSSSCG00000010303	SYNPO2L	4.24945
ENSSSCG00000024919		4.17652
ENSSSCG00000017500	TCAP	4.14783
ENSSSCG00000015617	G0S2	4.10478
ENSSSCG00000024676	SRPK3	4.08754
ENSSSCG00000028299	ACAA	3.87187
ENSSSCG00000015835	DUSP26	3.80312
ENSSSCG00000018073		3.80085
ENSSSCG00000025596	C1orf170	3.79457
ENSSSCG00000008200	ANKRD23	3.78233
ENSSSCG00000029000	SMPX	3.76134
ENSSSCG00000004918	ALPK2	3.74639
ENSSSCG00000022209	ITGB1BP2	3.72745
ENSSSCG00000020785	DES	3.71415
ENSSSCG00000028773	DLK1	3.69125
ENSSSCG00000014047	FGFR4	3.63092
ENSSSCG00000011238	ARPP21	3.63057
ENSSSCG00000029199	SCN4B	3.53253
ENSSSCG00000011508	LMOD3	3.53118
ENSSSCG00000001804	HOMER2	3.48905
ENSSSCG00000013916	COMP	3.44801
ENSSSCG00000007231	MYLK2	3.42651
ENSSSCG00000016215	TUBA4A	3.42480
ENSSSCG00000010603	NEURL1	3.36644
ENSSSCG00000006745	CASQ2	3.35000
ENSSSCG00000005938		3.32191
ENSSSCG00000024342	AQP4	3.23793
ENSSSCG00000002007	FITM1	3.16681
ENSSSCG00000012667	IGSF1	3.14445
ENSSSCG00000021345		3.12620
ENSSSCG00000024011		-8.12835
ENSSSCG00000021003	Metazoa_SRP	-4.87460
ENSSSCG00000030522		-4.81796
ENSSSCG00000028674	CR2	-4.55966
ENSSSCG00000015294	CR1	-4.42994
ENSSSCG00000026386	SLC30A2	-4.26379
ENSSSCG00000001570	PI16	-3.77443
ENSSSCG00000015663		-3.71187
ENSSSCG00000023591	GPR111	-3.59190
ENSSSCG00000025858		-3.57433
ENSSSCG00000002368	LTBP2	-3.52004
ENSSSCG00000025578	ALDH1A2	-3.30958
ENSSSCG00000017702	CCL23	-3.24031

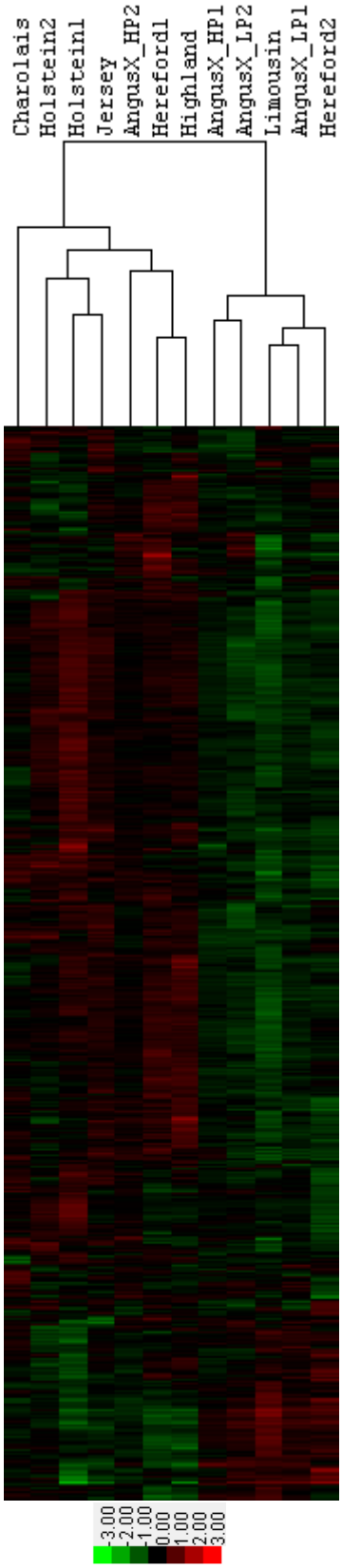
ENSSSCG00000005638	LCN2	-3.22515
ENSSSCG00000007501	BMP7	-3.18143
ENSSSCG00000013556	EMR1	-3.18044
ENSSSCG00000023542		-3.17486
ENSSSCG00000025698	SERPINE1	-3.11933
ENSSSCG00000030655		-3.03108
ENSSSCG00000021222		-2.93687
ENSSSCG00000011683	PCOLCE2	-2.92698
ENSSSCG00000009332	TEX26	-2.91707
ENSSSCG00000003414	ANGPTL7	-2.86092
ENSSSCG00000016628	WNT2	-2.83448
ENSSSCG00000016129	GPR1	-2.79065
ENSSSCG00000006172	PI15	-2.78196
ENSSSCG00000004261		-2.75323
ENSSSCG00000008737	C1QTNF7	-2.72805
ENSSSCG00000016684	SCRN1	-2.72250
ENSSSCG00000003521	WNT4	-2.71442
ENSSSCG00000007529	SYCP2	-2.70044
ENSSSCG00000000252	KRT8	-2.66667
ENSSSCG00000011472	DNASE1L3	-2.63083
ENSSSCG00000011625	CHST13	-2.62246
ENSSSCG00000005309	FAM166B	-2.61160
ENSSSCG00000006073	OSR2	-2.58528
ENSSSCG00000007435	PLTP	-2.55981
ENSSSCG00000017358	SLC4A1	-2.55267
ENSSSCG00000017367	MPP2	-2.52974
ENSSSCG00000000660	A2M	-2.52713
ENSSSCG00000027549		-2.51359
ENSSSCG00000022370	TNFSF9	-2.50188
ENSSSCG00000022728	HOXC10	-2.49776
ENSSSCG00000024439	PTGER4	-2.48921
ENSSSCG00000013551		-2.46146
ENSSSCG00000021119		-2.44137
ENSSSCG00000016312		-2.39581
ENSSSCG00000000182	WNT10B	-2.39370
ENSSSCG00000008606		-2.38299
ENSSSCG00000012137	BMX	-2.36057

Appendix 5

Top 100 up and downregulated DEG in AT of Female African elephants 25 versus 32 years old (FC ≥ 2, FDR < 0.05)

Ensembl ID	Gene symbol	logFC
ENSLAFG00000006514	SPP1	5.18305
ENSLAFG00000003799	DGAT2	3.66268
ENSLAFG00000012759	ECHDC1	3.56009
ENSLAFG00000009790	TNFAIP6	3.55061
ENSLAFG00000022714	THRSP	3.31822
ENSLAFG00000017790	SCD	3.16670
ENSLAFG00000010664	FBP2	3.15192
ENSLAFG00000009787	MMRN1	2.99543
ENSLAFG00000011486	STMN2	2.92353
ENSLAFG00000016209	TYR	2.90826
ENSLAFG00000010714	LEP	2.71302
ENSLAFG00000022206	FABP3	2.67559
ENSLAFG00000005002	DBI	2.61890
ENSLAFG00000015005	S100A5	2.61088
ENSLAFG00000012451	REXO2	2.54508
ENSLAFG00000003267	NEXN	2.51137
ENSLAFG00000010885	TAGLN3	2.48138
ENSLAFG00000021532	ELOVL6	2.37837
ENSLAFG00000006155	ACSM3	2.33986
ENSLAFG00000027812		2.31105
ENSLAFG00000014935	S100B	2.29584
ENSLAFG00000032759	AGT	2.28994
ENSLAFG00000011855	NDUFA4	2.20651
ENSLAFG00000002078	LOC100669935	2.19588
ENSLAFG00000009874	FAM195A	2.16364
ENSLAFG00000005140	ME1	2.15508
ENSLAFG00000010483		2.15348
ENSLAFG00000004468	FAM213A	2.10527
ENSLAFG00000026313	ATP5I	2.09921
ENSLAFG00000007372	FDFT1	2.09685
ENSLAFG00000000591		2.09527
ENSLAFG00000017506	CIDEC	2.08964
ENSLAFG00000010960	RARRES1	2.07946
ENSLAFG00000028777		2.07484
ENSLAFG00000000057	LOC100667282	2.06757
ENSLAFG00000012805	TCEA3	2.06732
ENSLAFG00000013675	RASGRP3	2.06169
ENSLAFG00000006157		2.05214
ENSLAFG00000016297	DNAJC28	2.05089
ENSLAFG00000032757	SMIM11	2.04820
ENSLAFG00000002952	PTPLB	2.03852
ENSLAFG00000027252		2.03711
ENSLAFG00000023227	FAM198A	2.02927
ENSLAFG00000000399	UGP2	2.02415
ENSLAFG00000021917	FILIP1L	2.01040
ENSLAFG00000018352		2.00796
ENSLAFG00000009910	NSA2	1.99705
ENSLAFG00000007901		1.98953
ENSLAFG00000008171		1.98313
ENSLAFG00000015397	MME	1.98239
ENSLAFG00000008834	FOSB	-6.05516
ENSLAFG00000001888	NFKBIZ	-4.37042
ENSLAFG00000007199	CETP	-4.15594
ENSLAFG00000025968	SOCS3	-3.87733
ENSLAFG00000029435	FOS	-3.49136
ENSLAFG00000007166	SELE	-3.29389
ENSLAFG00000021795		-3.20554
ENSLAFG00000022930		-3.17407
ENSLAFG00000029625		-3.13544
ENSLAFG00000027353	BTBD19	-2.97691

ENSLAFG00000003820	SEMA5A	-2.93275
ENSLAFG00000001043	DLL1	-2.92826
ENSLAFG00000033167		-2.89734
ENSLAFG00000012698	SGK1	-2.80305
ENSLAFG00000017323	IER3	-2.78410
ENSLAFG00000031616		-2.76331
ENSLAFG00000028819		-2.71053
ENSLAFG00000015516	ABCA1	-2.69126
ENSLAFG00000008944	GLI3	-2.65156
ENSLAFG00000012900	JUN	-2.61205
ENSLAFG00000031517		-2.58833
ENSLAFG00000009900	ID1	-2.56919
ENSLAFG00000011286	ZNF618	-2.55724
ENSLAFG00000030249	KIAA0754	-2.51481
ENSLAFG00000032802		-2.50968
ENSLAFG00000030265	SNAI1	-2.50933
ENSLAFG00000014805	KMT2A	-2.50868
ENSLAFG00000008530	JUNB	-2.50356
ENSLAFG00000009420	OSR2	-2.49090
ENSLAFG00000033042		-2.48944
ENSLAFG00000029310	FBN3	-2.47680
ENSLAFG00000006799	TMEM132E	-2.45619
ENSLAFG00000000421	KLF4	-2.45204
ENSLAFG00000008964	NFAT5	-2.43940
ENSLAFG00000016505	EGR1	-2.42101
ENSLAFG00000009180	PEAK1	-2.40966
ENSLAFG00000004367	RC3H1	-2.39391
ENSLAFG00000012514	FAM84A	-2.38837
ENSLAFG00000012857	PDPR	-2.38398
ENSLAFG00000022979	CYR61	-2.35814
ENSLAFG00000021534	KIAA1683	-2.33792
ENSLAFG00000018401	SOX4	-2.32943
ENSLAFG00000026357	AHNAK2	-2.29776
ENSLAFG00000005723	KDM7A	-2.29738
ENSLAFG00000011658	IGF1R	-2.29544
ENSLAFG00000000281	TCAP	-2.29416
ENSLAFG00000008102	PTPN14	-2.29383
ENSLAFG00000018327	DUSP6	-2.27481
ENSLAFG00000011961	NOTCH2	-2.25731
ENSLAFG00000029841	MAPK8IP1	-2.25577



Appendix 6

Hierarchical clustering of global gene expression detected through RNA-Seq in perirenal AT of dairy and beef breeds. Heat map shows genes differentially expressed between breeds, where red represents genes with higher mRNA expression, and green represents genes with lower mRNA expression. 4,496 genes from the total of 24,616, satisfied the initial QC criteria of all replicates with 50 read counts, and FC $\geq$ 2. Beef breeds included AngusX, Charolais, Hereford, Highland, and Limousin; while dairy breeds were Holstein and Jersey. Results showed that gene expressions from the replicates are specifically clustered into respective beef or dairy breeds. (AAX n = 4, Charolais n = 1, Hereford n = 2, Highland n=1, Holstein n = 2, Jersey n = 1, Limousin n = 1).

Appendix 7

Publications:

1. Nilsson, E. M., Fainberg, H. P., Choong, S. S., Giles, T. C., Sells, J., May, S., Stansfield, F. J., Allen, W. R., Emes, R. D., Mostyn, A., Mongan, N. P. & Yon, L. 2014. Molecular characterization of adipose tissue in the African elephant (Loxodonta africana). PLoS One, 9, e91717.
2. Choong, S. S., Fulton, J., Emes, R. D., Yon, L., Heery, D. M. & Mongan, N. P. 2015. Retinoids. In: Caplan, M.J. (ed.) Reference Module in Biomedical Science: Cancer and Endocrine Diseases. Elsevier.

Posters:

1. Choong, S.S., Mongan, N.P., Yon, L., Emes, R.D. Transcriptomics study of adipose tissue in different age group of Holstein cows. Poster session presented at: Society of Reproduction and Fertility Annual Conference; 2013 July 11 -13; Cambridge.
2. Choong, S.S., Mostyn, A., Yon, L., Mongan, N.P., Emes, R.D. Comparative transcriptomics in adipose tissue of Meishan & Large White pigs. Poster session presented at: Society of Reproduction and Fertility Annual Conference; 2014 Sep 1 -2; Edinburgh.