An examination of the molecular control of feed efficiency in beef cattle

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By

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Declaration

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Summary

Global agriculture is faced with the enormous challenge of feeding a rapidly growing population while being cognizant of the environmental footprint of such endeavours. A consequence of increased meat consumption worldwide is an increase in demand for animal feed. As feed provision is the single largest cost incurred by beef farmers, cattle with improved feed efficiency are central to profitable, sustainable and efficient beef production. A major constraint to genetic progress in feed efficiency is the difficulty and enormous expense of measuring it directly. Therefore, robust cost-effective molecular-based biomarkers for feed efficiency are necessary. This multidisciplinary thesis aimed to: (i) establish and physiologically characterise two populations of cattle, genetically and phenotypically divergent for feed efficiency as measured by residual feed intake (RFI); (ii) examine the premise that efficiency of energy transduction in cattle is a function of mitochondrial abundance and mitochondrial functional efficiency; (iii) examine the expression of genes involved in fatty acid metabolism in adipose tissue from animals divergent for RFI; and (iv) to examine the effect of RFI status, gender and diet as well as their interactions on targeted and global transcriptomic expression across three metabolically important tissues. The observations from this work suggest that firstly variation in mitochondrial number and/or enzymatic capacity may not be major contributors to variation in RFI. Secondly, the expression of *GLUT4* in adipose tissue is related to RFI and has potential to be investigated as a biomarker for RFI in cattle. Thirdly, transcriptomic analysis in liver tissue from bulls divergent for RFI, revealed evidence that efficient heifers had an enhanced capacity for mitochondrial function in muscle tissue, while oxidative response, protein mediation and cell signaling are likely to be processes that are influencing variation in feed efficiency in bull liver tissue. Finally, effects of RFI on gene expression in muscle of beef cattle are not consistent across breed type or dietary phase. In conclusion, while the results of this thesis provide a greater insight into the biological

mechanisms governing RFI in cattle, they challenge the practicality of developing a robust set of biomarkers for RFI that would be of ubiquitous utility to the beef cattle industry.

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Abbreviations

7SK RNA	7SK small nuclear RNA
ACACA	Acetyl-CoA carboxylase 1
ACAT1	Acetyl-CoA Acetyltransferase 1
ACLY	ATP Citrate Lyase
ACTA2	alpha smooth muscle actin
ADG	average daily gain
ADP	Adenosine diphosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ANT-1	ADP,ATP carrier protein, mitochondrial precursor
AT	Adipose tissue
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BF	Backfat
βHB	beta-hydroxybutyrate
BIF	Beef Improvement Federation
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BW	Bodyweight
cDNA	complementary DNA
CH	Charolais
CNS	Central nervous system
CO2	Carbon dioxide
COX I	Cytochrome c oxidase subunit I
COX II	Cytochrome c oxidase subunit 2

COX III	Cytochrome c oxidase subunit 3
CRELD2	CRELD2 cysteine rich with EGF like domains 2
CS	Citrate synthase
Ct	cycle threshold
CI	Complex 1
CII	Complex 2
CII	Complex 3
CIV	Complex 4
CV	Complex 5
CYTB	Cytochrome b
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBP	D site-binding protein
DPBS	Dulbecco's phosphate buffered saline
DCPIP	2,6-dichlorophenolindophenol
DEG	differentially expressed genes
DM	dry matter
DMI	dry matter intake
DNA	Deoxyribonucleic acid
DNAJB11	DNAJ homolog subfamily B member 11
DUB	Decylubiquinone
EBV	Estimated breeding value
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EIF2	Eukaryotic Initiation Factor 2
ELOVL6	Elongation of very long chain fatty acids protein 6
ER	Endoplasmic reticulum
ETC	Electron transport chain

FABP4	fatty acid-binding protein 4
FADH2	flavin adenine dinucleotide
FA	Fatty acids
FASN	Fatty acid synthase
FDR	False discovery rate
FE	Feed efficiency
FFA	Free fatty acids
GAO	Gross agricultural output
GC	Guanine cytosine
GH	Growth hormone
GIT	Gastro intestinal tract
GLUT4	insulin-regulated glucose transporter 4
GMPPB	Mannose-1-phosphate guanyltransferase beta
GNPNAT1	Glucosamine-Phosphate N-Acetyltransferase 1
GO	Gene ontology
GSH	Glutathione
GSTT1	Glutathione S-transferase theta-1
GSTTP1	Glutathione S-transferase theta pseudogene 1
GTT	Glutathione S-transferase 2
GWAS	Genome wide association study
H1	High concentrate 1 (phase 1)
H2	High concentrate 2 (phase 3)
H202	Hydrogen peroxide
HCA	hierarchical cluster analysis
HCL	Hydrochloric acid
HF	Holstein Friesan
Hi	High RFI

HIF	Heat increment of feeding
HMG-CoAR	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HMG-CoAS	3-hydroxy-3-methyl-glutaryl-coenzyme A synthase
HSP70	70 kilodalton heat shock proteins
HSPA5	78 kDa glucose-regulated protein precursor
HSPA6	Heat shock 70 kDa protein 6
HYOU1	Hypoxia up-regulated protein 1 precursor
IGF-1	Insulin-like growth factor 1
ILK	Integrin-linked kinase
IMM	Inner mitochondrial membrane
INSIG1	Insulin-induced gene 1 protein
IPA	Ingenuity pathway analysis
KCL	Potassium chloride
KCN	Potassium cyanide
KDE	Kernel density estimation
KEGG	Kyoto Encyclopedia of Genes and Genomes
KH2PO4	Potassium phosphate monobasic
KRT75	Keratin, type II cytoskeletal 75
LIMS2	LIM Zinc Finger Domain Containing 2
Lo	Low RFI
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MBW	Metabolic body weight
MDS	Multidimensional scaling
ME	Metabolisable energy
MR	Milk replacer
mRNA	messenger RNA
mtDNA	mitochondrial DNA

mtTFA	mitochondrial transcription factor A
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
ND5	NADH dehydrogenase, subunit 5 (complex I)
ND6	NADH dehydrogenase, subunit 6 (complex I)
NDF	Neutral detergent fibre
nDNA	nuclear DNA
NEFA	Non esterified fatty acids
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
O	Oxygen
PCA	Principal component analysis
PCR	Polymerase chain reaction
PGC1-a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARGC1a	Peroxisome proliferator-activated receptor gamma
PPARs	Peroxisome proliferator-activated receptors
PRDX3	Thioredoxin-dependent peroxide reductase
QH2	Ubiquinol
RC	Respiratory chain
RCR	Respiratory control ratio
RFI	Residual feed intake
RFLPO	60S acidic ribosomal protein P0
RIN	RNA integrity number
RNA	Ribo nucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative PCR

SCAT	Sub cutaneous adipose tissue
SCD1	Stearoyl-CoA desaturase-1
SCFAs	Short chain fatty acids
SIRT1	silent mating type information regulation 2 homolog 1
SLC16A1	solute carrier family 16 member 1
SM	Simmental
SNPs	single nucleotide polymorphisms
SOD	Superoxide dismutase
SREBPCs	Sterol regulatory element-binding proteins
STAR	Spliced Transcripts Alignment to a Reference
TAG	Triglycerides
TBP	TATA-box binding protein
TNB	2-Nitro-5-sulfanylbenzoic acid
TOP2B	DNA topoisomerase 2-beta
TRIS	tris(hydroxymethyl)aminomethane
UCPs	Uncoupling proteins
UPC2	Uncoupling protein 2
UPC3	Uncoupling protein 1
UTX2	X-linked H3K27 demethylase
VDAC	Voltage-dependent anion channel
VFI	Voluntary feed intake
VLDLs	Very low-density lipoprotein
VST	variance-stabilizing transformation
WAT	white adipose tissue
ZG	Zero grazed grass (phase 2)

Publications, presentations and awards

Conference proceedings

1. McKenna C. et al. (2016) Mitochondrial abundance and respiratory chain activities in tissue of cattle divergent in RFI. EAAP 2016
2. Mckenna C. et al. (2016) Relationship between mitochondria and compensatory growth in Holstein Friesian bulls. EAAP 2016
3. McKenna C. Et al. (2016) An examination of the biochemical control of feed efficiency in beef cattle. Walsh Fellowship seminar 2016
4. McKenna C. et al. (2017) Divergence in RFI effects lipogenesis in adipose tissue of beef cattle. ISAG 2017

Presentations

1. McKenna C. et al. (2016) Mitochondrial abundance and respiratory chain activities in tissue of cattle divergent in RFI. EAAP 2016
2. Mckenna C. et al. (2016) Relationship between mitochondria and compensatory growth in Holstein Friesian bulls. EAAP 2016

Posters

1. McKenna C. Et al. (2016) An examination of the biochemical control of feed efficiency in beef cattle. Walsh Fellowship Seminar 2016

Awards-Overseas Training

During my PhD programme I was awarded a scholarship to travel to Arkansas, United States. During this period I worked under the supervision of Dr. Walter Bottje, whose research programme is concerned with the molecular control of feed efficiency in poultry. I gained valuable experience in western Blotting, qPCR and bioinformatics analyses. This period allowed me to further develop my laboratory and data analysis skills while also nurturing a culture of collaboration.

Chapter 1

CHAPTER 1. INTRODUCTION TO LITERATURE REVIEW

The objectives of this literature review are to (i) provide a short description of feed efficiency (FE) and its importance in the beef industry; (ii) outline factors contributing to variation in feed intake; (iii) briefly review measures of FE with a specific focus on residual feed intake (RFI); (iv) outline the known physiological factors contributing to variation in RFI with a strong emphasis on mitochondrial function and dysfunction and lipogenesis; and (v) summarise the known genomic and transcriptomic information relative to RFI and give a short description of these technologies.

1.2 FEED EFFICIENCY

Worldwide agriculture is faced with the enormous challenge of feeding a growing human population in an economically and environmentally sustainable manner [1]. This growing global demand for meat has resulted in an increased demand for animal feed in the form of cereal grains, which has become a contentious issue [1]. Unlike pigs and poultry, on a life-cycle basis, cattle have an advantage in their ability to consume large amounts of plant resources that humans cannot, and subsequently convert these resources into meat [2]. Despite this, relative to pork or poultry, meat production by ruminants is less efficient due to the high cost per kilogram of beef. Furthermore, in beef production systems, animal feed provision represents up to three-quarters of total direct costs and, with the increasing cost of feed it will remain so for the foreseeable future, therefore making it a major factor in determining competiveness and profitability [3]. With this in mind, improvement in the FE of animals has the potential to

improve profits for the cattle producer while decreasing the environmental footprint of beef cattle production thus attempting to address the challenges of increasing feed costs and land pressure [4]. Increasing profits can be achieved by reducing production costs without compromising level of output [5]. Feed efficiency is the measure of how efficiently an animal converts its feed to product (carcass/meat). Feed utilisation is of great economic importance to beef producers as it is predicted that 65% -75% of total dietary energy intake of an individual animal is used exclusively for body maintenance. Animals with better FE consume less feed for the same production and are therefore more profitable and have a lower environmental footprint.

Unlike the pig and poultry industry, historically in the beef industry strong emphasis has been placed on improving output-related traits (weight and carcass) through genetic selection. Subsequently little emphasis was placed on cost related traits including feed intake, FE and or feed utilisation traits associated with output traits [6]. This is most likely due to the high cost and logistics associated with directly measuring feed intake in cattle. The challenge is to reliably and cost-effectively identifying these feed efficient animals and proliferating their genetics through animal breeding programmes. There is, thus, a critical necessity to develop robust cost-effective molecular-based biomarkers of FE.

1.3 FEED INTAKE AND FACTORS AFFECTING FEED INTAKE

Dry matter intake (DMI) is one of the most important factors in ruminant animal production, dictating the amount of nutrients available for production and thereby dictating gross feed conversion efficiency [7]. When trying to understand the functions that control DMI, ruminant animals present difficulties. Physiological factors such as feed intake are controlled by multiple factors. The majority of domesticated ruminant animals have feed available at all times. Despite

this fact, DMI is the primary limitation on production [7]. Factors that affect feed intake should be assumed to function with multiple interactions. Feedback regulators such as distension, protein and energy should be viewed as interacting processes when considering control of voluntary feed intake (VFI) [8].

1.3.1 Distension or Fill Feedback and Metabolic Control of Feed Intake

One of the most important factors controlling VFI is that of physical feedback. Distension or "fill" feedback happens when stretch and tension receptors within the rumen wall signal the degree of "fill" to the brain [8] . As an animal feeds, the increasing volume of rumen digesta leads to distension in the rumen wall, stimulating the distension receptors responsible for decreasing feed intake. As this is happening, rumen motility is amended increasing the flow of digesta from the rumen into the small intestine. This activity counteracts the stimulation of the distension receptors leading to increased feed intake once again [9]. This digesta has chemical properties such as protein and lipostatic feedback, and metabolites arising from this digesta such as glucose, fatty acids (FAs) and amino acids also contribute to feed intake in ruminants [9]. Physical feedback is generally responsible for the short-term regulation of VFI while metabolic regulation of VFI appears to be related to satiety over longer periods [9].

1.3.2 Dietary Factors

The dietary energy content of feed is an important factor in feed intake [10]. Forage, a fibrous and substantial feed source, has low digestibility, high fibre and a slow rate of passage and is relatively low in energy content compared to high concentrate diet which is generally low in fibre but is more digestible with a higher energy content [9]. This would indicate that VFI of forage diets is controlled by physical factors such as rumen fill and digesta passage [10] and VFI of high concentrate diets is controlled by metabolic factors such as the end products of rumen fermentation propionate and acetate [9-11]. Neutral detergent fibre (NDF) is related to

rate of digestion giving it an important role in regulating VFI [12]. Rumen distension due to fill has been associated with the NDF portion of the diet due to its low digestibility and slow rate of passage through the rumen. If feed has an increased NDF content then DMI is decreased [11] due to the low digestibility of NDF causing distension of the gastro-intestinal tract (GIT), which physically limits intake until the digesta moves further down the tract. Further research on identifying dietary factors that control VFI is required so that these constraints may be minimised.

1.3.3 Hormonal control of Feed intake and the Central Nervous System

Changes in intake are heavily influenced by alteration in physiological requirements of the animal. The homeostatic role of sensing nutrient intake and body reserves is conducted by the central nervous system (CNS). The CNS regulates short term control of energy intake and expenditure by integrating messages from the peripheral and central sensory pathways related to hunger and satiation. Long term energy balance is regulated through a complicated and integrated neuro-humoral system that dampens the impact of short-term changes in energy balance on metabolic reserves. This balance is achieved by a control system comprising metabolites and hormones that are secreted in proportion to the animal's energy status and metabolic reserves [13]. These metabolites and hormones will then interact with CNS targets influencing an appropriate physiological response [7].

Orexigenic (intake stimulating) and anorexigenic (intake-inhibiting) signals give an indication of metabolic state to the brain. These signals must reflect both the long-term energy stores (e.g adiposity, homeorhetic signals) and also the concentrations of key nutrients, metabolites and hormones in circulation that indicate the immediate energy status of the body (e.g. homestatic signals). These signals must fulfil certain criteria including i) circulating in either direct or inverse proportion to the degree of adiposity, ii) it must gain access to the brain and interact with receptors and transduction systems in neurons, iii) external administration or blocking

should affect VFI, iv) the secretion of orexigenic signals must follow a period of fasting and secretion of anorexigenic signals must follow a period of ingesting food and v) chronic infusions should alter body fat mass and the responsiveness of peripheral tissues to energy and adrenergic stimuli [7].

Insulin (anorexigenic) and glucagon (orexigenic) work in parallel with many metabolic hormones such as leptin [14] and ghrelin [15].

1.3.4 Animal factors involved in feed intake

Animal factors such as size, physiological stage, body composition and breed are related to VFI [9]. Body size influences VFI in that an animal with a larger size will have a higher maintenance requirement and therefore will need to consume more feed [12]. This is expressed on the basis of body weight raised to the power of 0.75 or otherwise known as metabolic body weight (MBW) and Huuskonen et al. (2013) observed that body weight (BW) was the most important predictor variable for DMI [16]. Feed intake stabilises as animals gain surplus fat and this may be due to a physical restriction on the capacity of the rumen by abdominal fat or by metabolic regulation such as higher levels of circulating leptin [12]. Previous research has shown that animal breed is associated with feed intake [12]. Dairy breeds such as Holstein-Friesian (HF) have a higher DMI when compared with dairy x beef breeds [17] and it was observed by [18] that late-maturing beef breeds had a lower DMI than HF.

1.4 MEASURING FEED EFFICIENCY

Animals utilise their consumed feed through a series of biological processes and interactions with the environment. This system is further complicated by the fact that feed intake is significantly correlated with body size and level of production. The body size of an animal is closely related to its energy requirements thus influencing feed intake (e.g the larger the animal, the larger the feed intake) [19]. In order to relate feed intake to production systems, many measures of FE have been accepted by the agricultural community, each differing in their application and benefits [20]. These measures are: gross efficiency, partial efficiency of growth, maintenance efficiency, cow/calf efficiency and residual RFI. In young beef cattle FE is evaluated in relation to growth. Methods used include partial efficiency of growth, feed conversion ratio (FCR) or its mathematical inverse feed conversion efficiency, and RFI. There is a high phenotypic and genetic correlation with these measures of FE [20]. Table 1.1 adapted from Arthur et al. (2008) summarises the various measures and terminology of FE giving a definition and formula for each measure [20].

Table 1.1 Definitions and formulas of feed efficiency measures.

Measure	Definition	Formula
Average daily gain (ADG)	Weight gain per day	Regression coefficient from the regression of weight on time (days)
Relative growth rate (RGR)	Growth relative to instantaneous size	100 X (log and wt –log start wt) Days on test
Feed conversion ratio (FCR)	Feed intake (FI) per unit wt gain	FI / ADG
Partial efficiency of growth (PEG)	Efficiency of wt gain not of maintenance food requirements	ADG/ FI – maintenance food (food maintenance requirements obtained from feeding standards.
Residual feed intake (RFI)	The difference between an animal's actual FI and its expected FI based on its size and growth. Independent of the level of production	FI –expected FI. Expected intake obtained by the regression of FI on average test period LWT and ADG

Adapted from Arthur et al. (2008) [20]

Current understanding indicates that RFI is moderately heritable and when animals are fed under an *ad libitum* regime RFI is phenotypically independent of growth traits and level of production [21, 22]. Selection for low RFI (high FE) will result in progeny that consume less food for the same production output as progeny of high RFI (low FE) animals. This review will focus on RFI and the biological and molecular processes which may contribute to variation in RFI.

1.4.1 Residual feed intake

The concept of RFI in beef cattle was first described and used by Koch et al. in 1963 [23]. Koch examined many different measures of efficiency recognising that feed requirements in growing cattle are dependent upon body weight of the animal and suggested that feed intake could be adjusted for BW, weight gain and/or any other production trait or energy sink identified [24]. This idea highlighted the two components of feed intake (1) expected feed intake for the given level of production and (2) a residual portion. This residual portion indicates an animal that deviates from their expected intake. Animals that eat less than expected and have a negative RFI value are considered to be high feed efficient or low RFI while animals that are assigned a positive RFI value are considered to be low feed efficient and are high RFI [24, 25]. RFI can be defined as the difference between an animal's observed feed intake and its expected feed intake for a given rate of growth [20, 24, 25]. Because RFI is phenotypically independent of production traits used to estimate it, selecting cattle for RFI is expected to decrease feed intake without significantly influencing growth rate and mature BW of the selected animals. Furthermore, previous research has demonstrated phenotypic differences in RFI in the region of 20% within populations of growing cattle [26, 27]. Therefore, it is assumed that RFI is a more precise and sensitive measurement of feed utilization since it is based on energy intake and energy requirements. As RFI is independent of production traits, this suggests that RFI represents differences in basic metabolic processes that may contribute to variation in efficiency

of feed utilization [20]. Previous research has revealed the presence of sufficient genetic variation for selection to increase efficiency of beef production [22, 28]. Research has suggested that RFI is moderately heritable across many breeds of beef cattle [29-31].

1.4.2 Statistical components of RFI

Calculation of a RFI value on a single animal uses a multiple linear regression of DMI on selected sources of significant energy expenditure or sinks [28, 32] as summarised below :

$$y = \beta 0 + \beta 1\ (ADG) + \beta 2\ (BW) + RFI$$

Where y is daily dry matter feed intake, 0 is the regression intercept, $\beta 1$ is the partial regression of daily intake on ADG and $\beta 2$ is the partial regression of daily intake on BW. Using this equation, RFI is expected to be independent of ADG and BW but is positively associated with DMI. This regression calculation also allows comparisons between individuals with different production potential during the measurement period [20] and can be adjusted for additional energy sinks to calculate DMI such as ultrasonic measures of fat and protein [33, 34]. When these are included as regressor variables it allows for the different energy demands of fat and protein accretion, or differences in the composition of ADG [35].

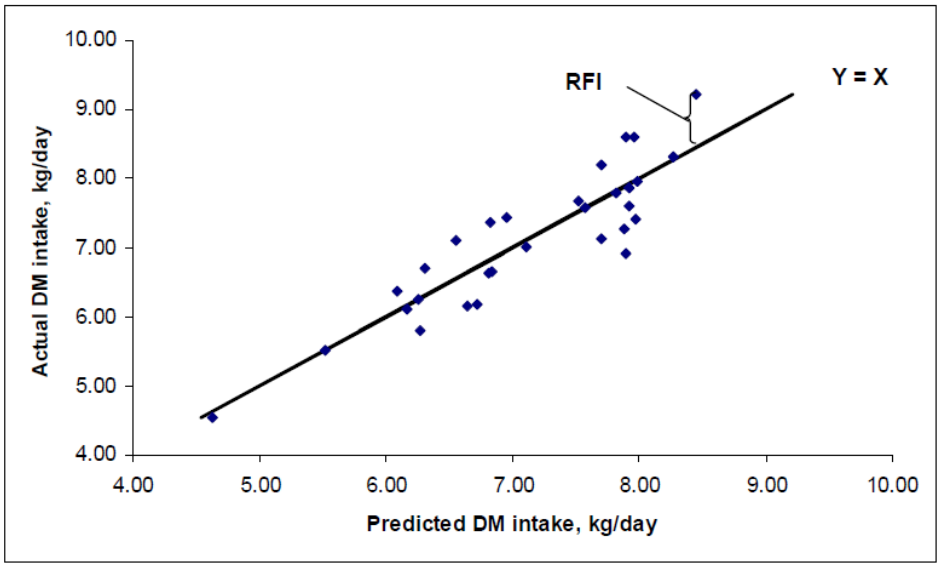

Figure 1.1. An example of the regression line used to calculate RFI.

Data points represent actual DMI and the regression line represents predicted DMI for a group of steers. Animals which have data points above the line are deemed inefficient and are ranked as high RFI as they consume more than their predicted DMI. Animals which have data points below the line are deemed efficient and are ranked as low RFI as they consume less than their predicted DMI [5]

1.4.3 Measuring RFI

Variation in feed intake in cattle cannot be ascertained from BW and level of production alone, therefore reliable measurement of feed intake is a necessary requirement in cattle evaluation systems [36]. Residual feed intake is an individual record for each animal taken over a long term feeding trial. The duration of test period necessary to calculate RFI varies between studies in the literature with some authors reporting test periods of 120 days [37] and others recommending 70 days [38] and even as low as 56 days with frequent automatic weighing [39]. A recent study concluded that intake evaluation periods can be shortened with minimal effects on the accuracy of predicting individual animal DMI [40]. The Beef Improvement Federation (BIF, 2010) suggests a 70 day test duration with live weight recorded at periodic intervals throughout the test period. The longer the test period the stronger the calculation of growth rate which appears to be the determinant of optimum test period [31, 37, 38, 41]. Animals must undergo an acclimatisation period of at least 21 days to adapt to test diet and test facilities and must be weighed on two consecutive days at the beginning and end of the test period [42]. As feed intake has been shown to be related to the age of animals when feeding tests are conducted, animals should have accurate birthdates assigned to them before entering a feed intake trial, so that age at the beginning of the test can be calculated. An animal should only undergo food intake measurement test after weaning but not younger than 240 days or older than 390 days. When measuring feed intake in a group, animals should have test start ages within a 60-day range.

1.4.3.1 Test Diets

Animals must have access to *ad libitum* feed during the test period to avoid data bias due to restricted feeding. All animals that are included in the one intake test should be fed the same diet. This diet should provide sufficient nutrients and energy to ensure expression of animal differences for production and intake. A sample of the test diet should be collected on a regular

basis and total diet chemical composition should be determined. Intake data should be reported on a DM basis to remove variability in moisture content across a diversity of diets. The majority of the intake evaluations performed in beef cattle have been conducted in cattle fed grain-based diets rather than those grazing forages In addition, regulation of feed intake is mainly driven by diet type, therefore there may be limitations of using feedlot intake information in development systems [40]. Durunna et al. (2011) hypothesised that diet type affects the measurement of FE when cattle are offered a growing and finishing diet in their respective growing and finishing phases [43]. Furthermore, regulation of feed intake may differ when cattle are fed different diets, and it is well known that DMI is related to energy content of feed or physical fill [12]. As DMI is an important component of FE, mechanisms of intake regulation for divergent diet types may confound the accuracy of comparing RFI in cattle when fed grain or forage [40]. A recent review highlighted a lower degree of variation in RFI is explained when animals were offered forage-based diets, and attributed this observation to the poorer intake characteristics and slower rate of passage through the rumen of a high forage diet [44]. There is a lack of knowledge comparing RFI values when cattle are fed differing diet type, however, a recent study found the correlation between forage and grain DMI to be 0.58 [40]. Furthermore, this study observed that this linear relationship of DMI closely paralleled the relationship of DMI during the growing and finishing period of grain-fed steers (0.56), suggesting that mechanisms of intake regulation on these diet types may not differ.

1.4.3.2 Breed effects

It is important to include animal effects such as breed in the calculation of phenotypic RFI. Schenkel et al. (2004) observed that when bulls were offered a high concentrate diet, Blonde d'Aquitane and Limousin breeds had the lowest RFI and Angus cattle had the greatest RFI with Charolais (CH), Simmental (SM) and Hereford breeds being intermediate [45, 46]. Limousin and Charolais were found to be the most efficient breeds, followed by Simmental, Hereford and

Angus as the least efficient in a study conducted by [22]. In a study conducted by Retallick et al. (2013) it was reported that Angus steers had a less desirable RFI than Simmental steers that were tested under the same conditions [47]. It was concluded by Herd et al. (2009) that breed or genetic makeup of an animal can influence variation in RFI [48].

1.4.3.3 Body Composition

Basarab et al. (2003) suggested that differences in fat deposition can account for 5-9% of the variation in DMI [49]. Previous work suggests that fat and muscle deposition are related to feed intake [45, 49]. It is becoming a common practice for research facilities to collect body composition measurements using ultrasound scanning. Thus, many studies include an adjustment for body fat in the statistical model computing RFI, which can increase variation explained from approximately 1 [50] to up to 5 per cent [51]. If body composition is considered when calculating RFI, measurements should be taken at least once during the feed intake test period [21].

1.4.4 Genetic component of RFI

RFI appears to be moderately heritable across a multitude of studies with heritability estimates ranging from 0.16 to 0.52 [20]. Differences of up to 20% in DMI between the RFI phenotypes have been observed after two generations of selection [29, 44], indicative of a strong underlying genetic basis, thus making it an attractive selection tool for feed efficient animals [35, 52]. This genetic variation has been demonstrated within populations and across multiple breeds of cattle [29, 41, 49]. The evidence that genetic variation in RFI exists, offers the potential that selection for high efficiency (low RFI) will produce off-spring that consume less, with no compromise in animal growth or performance [53].

1.4.5 Repeatability of RFI in beef cattle

In order to validate RFI as an economically important trait worthy of inclusion in selection programmes, an animal's status for the trait must be repeatable across various phases, physiological states and differing diets. Furthermore, high repeatability is very important for breeding programmes, where predictions of performance can only be made early in life. Knowledge of the repeatability of RFI where animals are consuming the same diet, during different stages of an animal's life and in different environments, is scarce [54]. Studies examining the repeatability of RFI in beef animals offered the same diet across two periods have found that RFI was repeatable in heifers (r = 0.62; $P<0.001$; [26]) and steers (r = 0.40; $P<0.10$; [55]). Moderate rank correlations (r = 0.52; $P<0.05$) have been observed between RFI in beef heifers offered the same diet across two consecutive periods [56]. However, Durunna et al. (2011) examined the repeatability of RFI between two consecutive periods for beef steers fed a grower diet, and subsequently a finisher diet, and observed that RFI was less repeatable (r = 0.33) [43]. Despite moderate repeatability, it is evident that animal re-ranking is occurring across different diets and stage of production which suggests the existence of a genotype x environment interaction in beef cattle [26, 43, 56]. Despite the studies referred to above, questions remain regarding the repeatability of RFI at different stages of an animal's life, on different diets, and in different environments. Positive phenotypic and genetic correlations have been reported between RFI measured in animals fed a grower diet and then subsequently measured on a finisher diet [28, 43, 57, 58]. These results indicate that RFI is moderately repeatable across different diets and stages of an animal's life, however they also point to evidence of a genetic x environment interaction. Recent studies have demonstrated that beef heifers ranked as low RFI under dry lot conditions remained low RFI when follow up pasture diet was offered [59-61]. In contrast, Manafiazer et al. (2015) observed that heifers ranked as low RFI on a high concentrate indoor feeding regime, had a lower DMI when subsequently

measured on a grazed herbage regime [59]. In addition, a recent study conducted by Coyle et al. (2017), concluded that only 50% of animals retained their RFI ranking when intake was measured across different diets and stages of production [62]. These discrepancies in repeatability of RFI may be explained by maturity, dietary energy content, the physiological state of the animal and the inherent difficulty in accurately quantifying herbage intake in grazing cattle [44, 60]. Thus, it is evident that some animal re-ranking occurs, suggesting existence of a genotype x environment interaction for RFI, and this is an important area for future research.

1.5 PHYSIOLOGICAL REGULATION OF RFI

Livestock follow the physical laws of conservation of mass and energy as was established in the late 19th century. It follows that variation in RFI must be controlled by measureable differences in biological processes and is the result of inherent variation in biological processes within an animal [63]. Most complex traits, including RFI, are characterised by complex interactions between cellular constituents such as DNA, RNA and proteins, and are usually influenced by multiple biological processes [64]. The biological mechanisms underpinning RFI have yet to be fully elucidated. It is hypothesised that there are at least 5 major processes that contribute to variation in FE, namely, 1) feed intake, 2) digestion of feed and associated energy use, 3) metabolism, 4) activity and 5) thermoregulation [65]. In a companion paper by the same authors the estimated contribution of various physiological mechanisms contributing to variation in RFI in beef cattle were reported (Figure 1.2) [66]. These were reported as digestibility (10%), activity (10%), body composition (5%), feeding patterns (2%), protein turnover, tissue metabolism and stress (37%), heat increment from fermentation (9%) and an un-accounted for portion (27%). Processes such as thermoregulation, metabolism and activity fall under the term “maintenance”, defined as the feed energy requirements for zero body energy [67]. Maintenance requirements, which are related to body size and level of production, dictate energy requirements of the animal and hence influence feed intake [67].

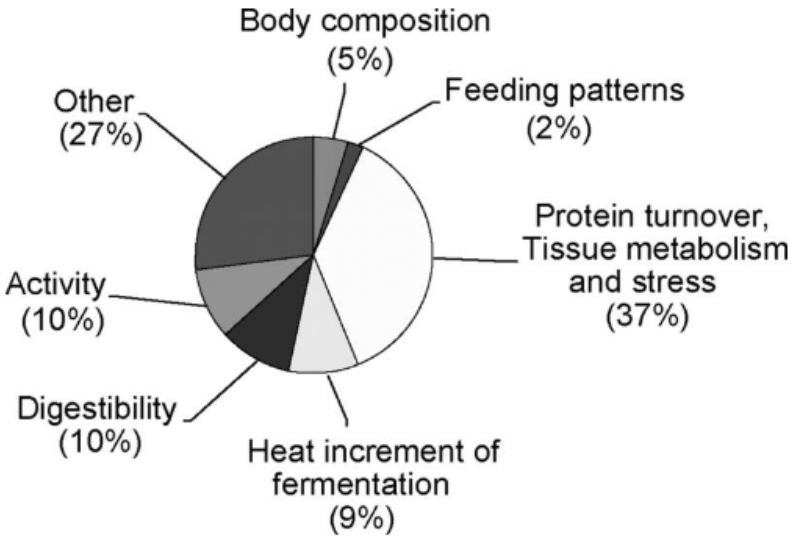

Figure 1.2 Contributions of various biological mechanisms to variation in RFI. It is estimated that digestibility (10%), activity (10%), body composition (5%), feeding patterns (2%), protein turnover, tissue metabolism and stress (37%), heat increment from fermentation (9%) and an un-accounted for portion (27%) are contributing to variation in RFI. (Adapted from Herd et al. (2009)[48]).

1.5.1 Feed intake

Variation in feed intake is related to variation in maintenance requirements of ruminants in that an increase in feed intake means an increase in the amount of energy expended to digest feed. This can be attributed to an increase in the size of digestive organs and also an increase in energy expenditure within various metabolic tissues. This is known as the heat increment of feeding (HIF) and is due to the work associated with the many physical and chemical processes involved in the mastication, digestion, absorption and metabolism of digested foods [68]. In ruminants the HIF accounts for approximately 9% of metabolizable energy (ME). As RFI is associated with differences in intake, it follows that the more efficient animals that eat less for the same level of production could be expending less energy as HIF [63].

Rate of ingestion and duration of the meal have been reported as key factors that determine the energy cost of eating in cattle [69]. It has been reported that low RFI animals settle into a regular feeding cycle faster than their high RFI counterparts who had more variable temporal patterns of feed intake early in the RFI test period [70]. RFI has been reported to be positively correlated with three feeding behaviour traits of feedlot steers, in that high RFI was associated with longer feeding time per day, more eating times per day, and faster rate of eating (g/min). In this study it was observed that feeding time and number of eating sessions also had positive genetic correlations with RFI [71]. Additionally, Fitzsimons et al. (2014) showed that low RFI animals spent less time engaged in feeding behaviour related activities [72].

1.5.2 Digestion and Methanogensis

The reticulo-rumen provides the profile of nutrients available for absorption in cattle [73]. The rumen is the location in which approximately 90% of NDF [74] and between 25-100% of starch [75] digestion occurs. Ruminant digestion consists of a number of processes; initially feed ingested is broken down by mastication and rumination. It is then subjected to further

breakdown by the microbial population within the rumen [76]. Passage rate of digesta through the rumen dictates the amount of time feed is retained for digestive action and the rate and potential extent of degradation dictates the digestion that can occur during the retention time [77]. As the level of feed intake relative to maintenance increases, the digestion of the feed decreases. In addition to the variation in intake there is also genetic variation in total tract digestion of feed. Richardson et al. (1996) reported that young bulls and heifers divergent for RFI differed by 1% in their ability to digest DM when fed a pelleted ration with a DM digestibility of 68%, accounting for 14% of the difference in intake between the two phenotypes [78]. The same authors observed a correlation ($r = 0.44$) between digestibility and RFI of cattle fed a high grain ration indicating that differences in digestibility accounted for 19% of the phenotypic variation in RFI. The authors concluded that less RFI or greater efficiency was associated with greater digestibility [79]. It is noteworthy that accurately measuring small differences in digestibility is difficult and assigning variation in digestion as a major factor explaining differences in RFI in beef cattle must be done with caution [77]. A recent study observed no differences in digestibility between high and low RFI beef heifers [27]. Additionally, studies in monogastrics deduce that differences in digestibility are not important sources of variation in RFI. Basarab et al. (2013) hypothesised that the conflicting results of studies examining the effect of RFI on diet digestibility in beef cattle most likely arises from differences in feed intake as opposed to any real differences in ability to digest feed [80]. From the majority of studies conducted to date, few obvious differences in rumen fermentation variables measured are evident between high and low RFI cattle, and where variation was observed, the results are equivocal and warrant further experimentation. For example, some data have suggested that low RFI cattle have lower concentrations of the volatile fatty acid propionate and a lower acetate:propionate ratio in ruminal fluid compared to those of high RFI [27, 60]. However, Krueger et al. (2009) found the opposite [81]. Kong et al. (2016) reported

no differences in the expression of genes involved in VFA metabolism [82], consistent with many studies that measured the physiological aspects of VFA metabolism [83, 84].

Feed consumed by ruminants primarily consists of fibrous forage which is composed of polysaccharides such as cellulose and hemicellulose [12]. Enzymes synthesised by the microbes present in the rumen break down and digest these polysaccharides converting them to short-chain fatty acids (SCFAs) that can be utilised by the animal for energy production [76, 85]. Methane is a by-product of this process. These SCFAs are absorbed through the rumen wall and fulfil the energy needs of the animal; methane is not absorbed and is emitted to the atmosphere together with its retained energy contributing to energy lost from the feed as well as global warming [86]. There is evidence to suggest a relationship between the rumen microbiome, VFA and RFI [87, 88]. Furthermore, differences between high and low RFI animals have been reported in terms of methane production and microbial composition in the rumen [27, 86, 89, 90]. These results indicate that differences in the processes of digestion, microbial fermentation and substrate availability could provide a possible mechanism to explain variation in efficiency of feed utilization [63].

1.5.3 Body composition

The energetic cost of deposition of a unit of fat is much greater than the cost of an equivalent unit of muscle [77]. However, the energy requirements to maintain existing fat depots are significantly less than that of protein [91]. Furthermore, there is more inter-animal variation in the efficiency of protein turnover in lean muscle compared to fat, most likely due to differing rates of protein turnover and degradation between organs and tissue [44]. Accordingly, there are both energy storage and energy turnover issues to consider and any variation in composition of gain, and in composition of the body, can influence the apparent efficiency of nutrient utilisation [63, 91]. At present, there is much equivocation in the published literature on body composition status in relation to FE. It has been suggested that high RFI animals have a higher

rate of protein degradation in muscle and liver and a less efficient mechanism for protein deposition and there may be a genetic origin for this trait [79]. Many studies that did not include BF in the model used to predict RFI have shown that high RFI animals tend to be fatter while low RFI animals tend to be leaner [34, 92]. In contrast, other studies have reported no relationship [55, 93-95] However, where others have included adjustments for BF in the model, a trend for a positive relationship [34], no relationship [92], or a negative relationship [96] between RFI and measures of body fat were reported.

It appears that feed efficient animals may be leaner while growing but it has been proposed that once these animals are fully grown adults they may in fact be fatter than their less efficient counterparts. These same authors hypothesised that in young, growing heifers, those of low RFI may partition more energy into lean tissue accretion and then, later in life when they are more mature and not in growth phase, fat tissue accretion becomes prominent as there is a lower energy requirement for maintenance of this tissue relative to lean tissue [91]. Basarab et al. (2007) supported this notion when retrospective analysis on historical productivity records found that females which produced low RFI progeny had greater back fat thickness compared to those that produced high RFI progeny [97]. Despite this, other studies have found conflicting results, including Arthur et al. (2004) who found that high RFI cows had a greater rib fat thickness compared to low RFI cows at the start of the breeding phase but these differences disappeared during gestation [53].

1.5.4 Tissue metabolism and maintenance

Metabolism is a complex and highly organised process of cellular activity involving multiple enzymes, substrates, and pathways within the mammalian body. Metabolism has 3 main biological functions; a) obtain chemical energy via degradation of energy-rich nutrient molecules, b) repartition substrates to meet energy demands of tissues, and c) synthesize and degrade biomolecules needed for specialised cellular functions [98]. In cattle the estimated

proportion of total energy intake required solely for body maintenance is typically in excess of 50% in adult cattle, thus making it the single most important factor that determines metabolism efficiency [99, 100]. Given the large energetic requirement to maintain energy homeostasis, it is no surprise that a number of physiological and biochemical processes are involved, some of which are discussed below.

Maintenance of tissues and metabolic functions such as protein turnover and substrate cycling, of the GIT and liver, are responsible for substantial energy consumption [99]. From the literature, it is estimated that combined, the GIT and liver account for approximately 14% of body weight but account for up to 48% of total energy expenditure [67, 99, 101-105]. Muscle tissue accounts for 40-45% of empty body weight and approximately 21% of total body energy consumption, highlighting the disproportionate energy expenditure relative to weight [99]. Energy consumed by the GIT and liver is related to absorption and subsequent transportation of nutrients to tissues, where it is utilised for energy production but also for maintenance of tissue integrity and mass [105]. Due to the large proportion of energy expenditure in these different tissues, it follows that changes in the energy metabolism or size of these organs could have a large effect on efficiency of energy utilisation by the whole animal [101, 105] and it appears that increases or decreases in the weight of the liver and GIT are directly proportional to dietary intake [101].

Previously it was found that low RFI steers had a lighter liver, stomach and GIT when compared to their high RFI counterparts [49]. In addition, it was also observed that low RFI bulls had lower liver and kidney weight, however no other organ weights were associated with RFI in this study [106]. In contrast to this, multiple studies have also found no effect of RFI on the weights of organs in beef cattle [107-111]. These conflicting results indicate further work is required to elucidate the possible effects of RFI on the weight of organs and the energetic efficiency of biological processes within these tissues.

1.5.4.1 Energy consumption in muscle

Skeletal muscle can account for up to 40-45% of the total body mass of ruminants and when considering the whole animal, can contribute 60% or more to systemic metabolism [91]. When compared with other tissues skeletal muscle is considered one of the most energy consuming tissues and in its active state it must be supplied with energy rich substrates that can accommodate these metabolic needs [91]. Energy substrates that are utilised by muscle include glucose, triacylglycerol (TAG) and blood-borne free fatty acids (FFA), fatty acids (FA) and ketone bodies [112].

Glucose, a simple sugar, has two distinct fates in muscle metabolism. It is either oxidised via glycolysis or stored as glycogen [113]. Glycolysis is a process that occurs in the cytosol of cells to generate a small amount of adenosine triphosphate (ATP) [91]. Pyruvate (a product of glycolysis) is oxidised by the citric acid cycle generating the coenzymes nicotinamide adenine dinucleotide (NADH) and ubiquinol (QH_2). These coenzymes are oxidised and large amounts of ATP are formed during oxidative phosphorylation, occurring in the inner mitochondrial membrane (IMM) [114]. Glycogen is the main storage form of glucose in resting muscle and these stores can be called upon in times of increasing energy demand, being converted into glucose-6-phosphate for entry into glycolysis. Skeletal muscle has the ability to obtain glucose in the same manner via mobilisation of liver glycogen stores [91]. In times of high energy demand, glucose transport mediated by the insulin-mediated glucose transporter 4 (*GLUT4*) is the rate limiting step [115, 116]. An increase in *GLUT4* expression leads to a decreased rate of FFA oxidation and increased glycogen synthesis meaning that if the system is in a state of elevated glucose oxidation, it requires less FFA for the same level of demand [117].

Energy can be stored as TAG which are the most abundant form of lipids in the body. It is metabolically advantageous to store energy as TAG over carbohydrate (glycogen) as oxidation of TAG via mitochondria can generate up to twice as much energy as oxidation of

carbohydrates [91]. Muscle, liver and adipose tissue (AT) store TAG as a reservoir of metabolic fuel, which can be broken down and utilised by the muscle in the form of FA [98].

Blood-borne FFA of adipose origin are the major substrate used by skeletal muscle at times of rest and during low to moderate exercise over a sustained period of time [118, 119]. As energy demand increases, FFA are mobilised from their AT into plasma, followed by an increase in muscle uptake and oxidation of FFA. Plasma FFA can account for up to 90% of the muscle's lipid fuel requirements [112] with the rest coming from muscle FA [118, 120].

In times of severe starvation, ketone bodies are utilised as an alternative source of energy by muscle. Ketone bodies are formed from the incomplete oxidation of FAs to acetyl CoA in liver mitochondria, and skeletal muscle can then derive energy from the oxidation of ketone bodies to carbon dioxide (CO_2) within muscle mitochondria [121]. In these circumstances extreme substrate partitioning comes into play and only tissues deemed of high importance will be able to utilise energy generated from ketone bodies. While skeletal muscle can utilise ketone bodies, its energy demands may not be met in this instance [91]. Taking into account the underlying mechanisms in metabolism of muscle fibres, it is evident that variation in the utilisation of substrates by muscle could indeed contribute to variation in the whole energetic efficiency of an animal.

1.5.4.2 Energy substrate Partitioning and its role in variation in feed efficiency

The mammalian body has the capacity to selectively direct certain energy substrates for utilization by different tissues based on necessity and uptake efficiency of the tissue. This process is known as substrate partitioning and is related to metabolic state of the animal [91]. Energy substrate partitioning is modulated by three mechanisms. First, substrate mobilization occurs in the AT, liver and muscle, reversing energy storage and activating the processes of gluconeogenesis, lipolysis and glycogenolysis (see Figure 1.3). Next, substrate uptake occurs and finally substrate oxidation. Substrate oxidation is a tightly regulated process which

determines which substrates will be used. The rate at which substrates will be oxidized reflects the demand of the tissue thus making this the critical step of the substrate partitioning process [91].

Substrate partitioning is dependent on complex interactions of a range of extrinsic and intrinsic factors. Extrinsic factors affecting substrate utilization include temperature [122], stress, nutritional status, age, gender and exercise [91]. These extrinsic factors together with systemic (endocrine) and intracellular factors have vital influences in controlling substrate balance throughout an animal's lifetime [119, 120, 123, 124].

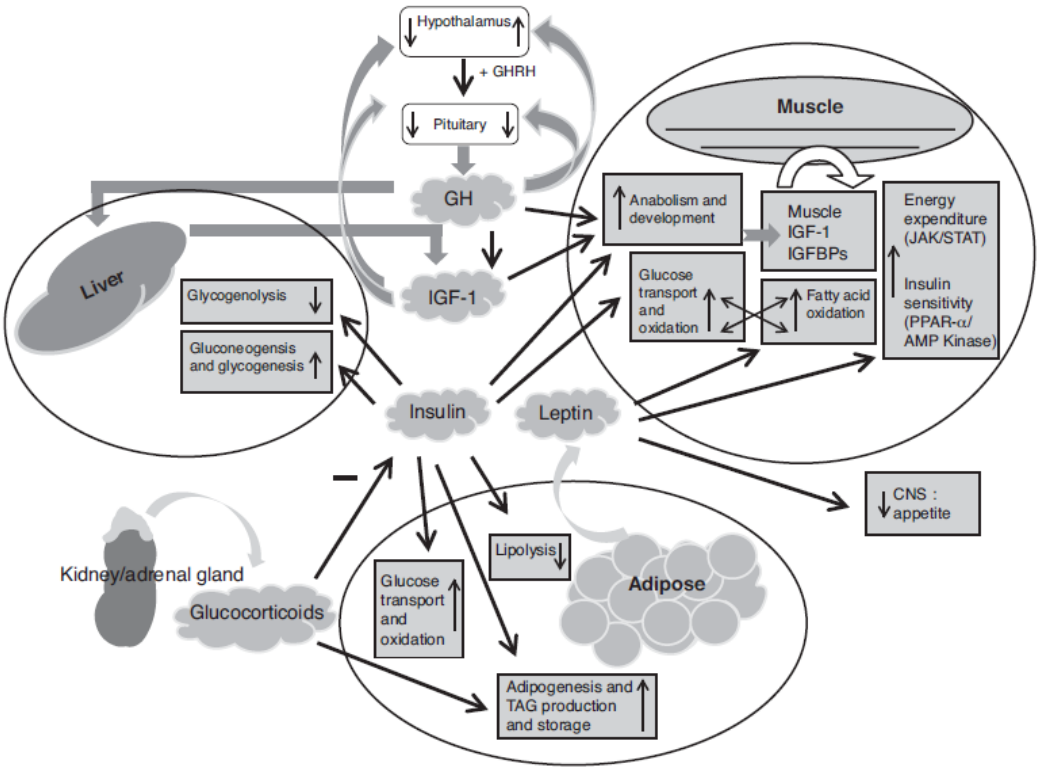

Figure 1.3. A brief overview of the pathways and processes that link metabolism of muscle, liver and adipose tissue that have the potential to contribute to variation in feed efficiency.

Broad, grey arrows link tissues to endocrine factors and endocrine feedback loops. Heavy, black arrows link endocrine signals to tissue-specific processes. Black arrows within process boxes show up or down regulation. The crossed arrows indicate the competing interactions of insulin and leptin that stimulate glucose oxidation and fatty acid oxidation, respectively. The broad white arrow indicates that stimulation of locally produced IGF-1 and IGF-binding proteins (IGFBPs) results in autocrine/paracrine signaling that also regulates anabolic processes in muscle. Taken from Welch et al. (2012) [91].

As seen in Figure 1.3 the hormones leptin and insulin appear to have antagonistic effect on energy partitioning in muscle, with insulin blocking oxidation of FFA and leptin suppressing insulin [125-127]. Leptin can directly stimulate FA oxidation in muscle by activating the 5-AMP-activated protein kinase (AMPK), which subsequently inactivates acetyl CoA carboxylase (facilitates fatty acid biogenesis) [128]. It has been proposed that leptin may stimulate thermogenesis in skeletal muscle via uncoupling proteins (UCPs) [129]. With this, leptin may facilitate a cycling between de novo lipogenesis and lipid oxidation, a process which requires glucose and thus interdependence on insulin signalling. Differences in leptin levels and subsequent differences in substrate partitioning could potentially contribute to variation in energetic (and feed) efficiency [91].

1.5.4.3 Adipose Tissue

Adipose tissue is a long-term fuel store possessing the ability to mobilise nutrients in times of deficiency[130]. There are different types of AT found in mammals; brown adipose tissue (BAT) and white adipose tissue (WAT). Different adipose depots are classed as either BAT or WAT and each one differs in its metabolic regulation [131, 132]. White adipose tissue includes visceral organ, subcutaneous, intermuscular and intramuscular fat. Visceral fat is found around the inner organs and is categorised as omental, mesenteric, retroperitoneal and pericardial. Subcutaneous fat is found abdominally and in the glutofermoral region. Brown tissue is found in the clavicle and subscapular region [131].

The variation in fat depot metabolism, adipocyte size, and physiological dynamics shows that animals vary in relative proportions of fat across different depots resulting in a variation in the energetic dynamics of their overall metabolism. Here lies a potential tissue-level difference in maintenance requirements which could contribute to variation in FE [91].

1.5.4.4 Adipose tissue and its potential contribution to variation in feed efficiency

White adipose tissue is the main reservoir for energy storage and regulates many processes related to feed intake, energy homeostasis and whole-body physiology [133, 134]. The response of AT to feed intake has been widely reported in different species, however, there remains a lack of knowledge on the relationship between adipose biological mechanisms and variations in feed utilization [135].

Leptin is a hormone found in the CNS which is produced primarily by WAT and its main role is the regulation of energy flow [136-139]. The central and peripheral actions of leptin result in a decrease in energy conservation and an increase in energy usage by partitioning substrates towards oxidation rather than storage [140-142]. Feeding and elevated blood glucose and glucocorticoid levels increase leptin gene expression; while fasting, insulin deficiency and high cyclic adenosine mono-phosphate levels will decrease leptin gene expression [142]. Deficiencies in leptin production or leptin receptors has been shown to result in animals exhibiting obesity among may other metabolic disorders [141].

It has been proposed that leptin is a regulator of appetite and energy partitioning, thereby implicating it as a regulator of FE [143]. As an animal reaches maturity, the majority of additional mammalian growth occurs in the form of AT, which in turn increases circulating leptin concentrations [144]. Studies in multiple species including swine [145], mice [146] and humans [147] have shown relationships between leptin gene polymorphisms and body composition. These studies indicate that polymorphisms in the leptin gene are a potential source for variation in RFI.

Previously, single nucleotide polymorphisms (SNPs) have been reported within exon 2 and the promoter regions of the bovine leptin gene [28, 148]. A mutation in exon 2 has been shown to impart a partial loss of biological function that is associated with a fatter carcass and an increase in leptin messenger (mRNA) levels [148]. Several studies have reported a relationship between

leptin and RFI [26, 149, 150]. Most recently it was reported that leptin mRNA levels were higher in subcutaneous AT of low RFI cattle when compared with their high RFI counterparts [151].

1.5.5 Lipid Metabolism

Extensive lipid metabolism occurs in the rumen, and this has a huge impact on the profile of FA available for absorption and tissue utilization. Lipid metabolism in the ruminant comprises a plethora of different processes that can be classified into two pathways of action. The first pathway is related to the ruminal biohyrdration of dietary FA, and their subsequent absorption and transportation to other tissues. The second pathway is related to the action of FA as metabolic modifiers, either by directly altering the synthesis and deposition of FAs in the tissues or by affecting biological processes in the animals including modulation of gene expression [152]. The starting point of lipid metabolism is the dietary intake of feedstuffs, which is followed by an extensive ruminal de-esterification and biohydrogenation of dietary lipids as well as synthesis of short-chain FA from dietary fibre compounds and fermentable carbohydrates. Fatty acids and their precursors are absorbed and further digestion and absorption of rumen-protected lipids occurs in the intestine. Upon absorption, FA and their precursors are transported to target tissues for further metabolism [152-154]. Cellular lipid metabolism, the next stage of lipid metabolism, comprises cellular uptake of FA and FA precursors via transport and hydrolysis, *de novo* fatty acid and triglyceride synthesis, intracellular lipid storage, lipid breakdown and fatty acid oxidation, transport and excretion processes and finally, modulation of gene expression [132, 155-158]. Detailed accounts of these processes are beyond the scope of this review and the reader is directed elsewhere for a more in depth explanation [159].

As aforementioned, lipids or FAs are major fuel sources. The supply of FA comes from circulating NEFA and lipoproteins which include chylomicrons and very low density

lipoproteins (VLDL). Large amounts of lipid can be deposited in AT because adipocytes are capable of both hypertrophy and hyperplasia and mature adipocytes contain 70-90% lipid depending on dietary energy supply [160]. Lipid metabolism refers to the synthesis and degradation of lipids, obtained from diet or synthesised by the liver, in cells. The amount of fat in AT is determined by rates of lipid synthesis (lipogenesis) and lipid degradation (lipolysis) [161]. The substrate pool of FA has three sources: cellular uptake from circulating lipoproteins (dietary derived), FA released from adipose depots and fatty acids synthesised de novo in the tissue [161].

Lipogenesis is a physiological process of endogenous FA synthesis that increases inversely to muscle tissue development; after ruminants reach genderual maturity, as muscle growth decreases and AT increases [162]. Further to this, it has been demonstrated that fat deposition is not homogeneous throughout an animal's body [163]. Ruminants are unique as lipogenesis occurs primarily in AT and to a small extent in the liver. This is in contrast to humans and swine where the liver is the primary site [164]. Following absorption of dietary FA or de novo synthesis of FA, TAG must be incorporated into the animal's AT [165].

Glucose and acetate are the main carbon sources for lipogenesis in ruminants and substrate specificity can differ with each depot site [166, 167]. Animal nutritional status is the main factor that controls the rate of fat deposition and acetate, a volatile fatty acid produced during ruminal fermentation is the main precursor for FA synthesis [168]. Following absorption across the ruminal epithelium and transport to peripheral tissues, acetate is converted to acetyl-CoA via enzymatic action by acetyl-CoA synthetase [169]. The combined presence of acetyl-CoA and reduced nicotinamide adenine dinucleotide phosphate (NADPH) is essential for de novo fatty acid synthesis. Lipogenesis takes place in the cytosol of the cell, therefore the acetyl-CoA produced in the mitochondria must be converted to citrate in order to cross the mitochondrial membrane into the cytosol. Once in the cytosol citrate is cleaved by the enzyme citrate lyase

into oxaloacetate and acetyl-CoA for subsequent lipogenesis. The metabolic processes that are involved in FA synthesis and their subsequent esterification to TAG have been reported in considerable detail [170]. Fatty acids are synthesised via an extramitochondrial system, which converts acetyl-CoA to palmitate in the cytosol. Glucose, which in ruminants, is obtained from gluconeogenesis and VFA, is utilised for the initiation of lipogenesis [170].

Pyruvate is formed from acetate (absorbed directly from the gut) via oxidation in the mitochondria and it is subsequently converted to acetyl-CoA which is the principal building block of FA. The biosynthesis of palmitate is catalysed by the enzyme complex fatty acid synthase (FASN). The system is active in liver, kidney, brain, lung, mammary gland and AT. The requirements of the system are reduced NADP, ATP, HCO_3- as a source of carbon dioxide, and manganese ions [12]. Palmitate is the final product of FA synthesis. The chain of palmitate may undergo elongation or it may be converted into unsaturated fatty acids through the action of the enzyme stearoyl-CoA desataurase (SCD1).

Many transcription factors are involved in lipid metabolism. Among these are peroxisome proliferator-activated receptors (PPARs) and Sterol regulatory element-binding proteins (SREBPs). PPARs are a family of nuclear receptors that bind to FAs and are crucial to the regulation of nutrient metabolism and energy homeostasis. There are three PPAR isoforms, α, β and γ, which differ in their target tissues, physiological properties and developmental stages of tissues [171, 172]. PPARγ is highly expressed in adipocytes and to a lesser extent in muscle [173] and plays an important role in the control of adipogenesis, lipogenesis and insulin sensitivity [174].

Lipids are absorbed in the small intestine and are transported throughout the body by plasma lipoproteins. In the liver FA can be utilised in four different ways depending on the metabolic state of the organ. They may be secreted as bile, oxidised completely to carbon dioxide or

partially to acetic acid or ketone bodies [175]. Fatty acids are oxidised in the peroxisomes and mitochondria (to acetyl-coA), contributing energy to the Krebs cycle [154].

Lipid metabolism and partitioning between different metabolic pathways in the liver is controlled by the fat: carbohydrate ratio and certain hormones, for example, insulin stimulates de novo lipogenesis and esterification of FA, but inhibits their oxidation [154].

Triglycerides and neutral fats constitute 90% of lipid fractions in adipose tissue. In the adipocyte the content of triglycerides depends on a balance between de novo synthesis of FA, FA uptake, FA esterification, lipolysis and re-esterification of FA produced during lipolysis. As already mentioned, in the ruminant, adipose tissue is responsible for approximately 90% de novo FA synthesis while liver is responsible for only 5% [130].

The importance of lipid metabolism in influencing RFI in cattle has previously been highlighted by [63]. In a recent study by Karisa et al. (2014) that analysed gene networks associated with variation in RFI, a major hub was associated with the insulin induced gene 1 (INSIG 1) [176]. This gene hub had interactions with many molecules related to energy, lipid and steroid metabolism and the authors suggest that polymorphisms present in these genes may influence body composition in beef cattle.

The same study observed that the metabolite creatinine was significantly associated with RFI [176]. Creatinine interacts closely with *AMPK,* an enzyme that has a vital role in cellular energy homeostasis. Stimulation of FA oxidation and ketogenesis in the liver, inhibition of cholesterol synthesis, lipogenesis, and triglyceride synthesis, inhibition of adipocyte lipolysis and lipogenesis, stimulation of skeletal muscle glucose uptake, and modulation of insulin secretion can all be attributed to the activation of *AMPK* [177].

In livestock species, cholesterol obtained from the feed is maintained at a relatively constant level, because of this it can be assumed that variation in the levels of cholesterol in the cell is

due to variation in cellular regulation of cholesterol biosynthesis [12]. Karisa et al. (2014) utilised biological network analysis to determine pathways that may be involved in variation in FE. From this analysis it was hypothesised that FE in beef cattle is associated with cholesterol levels in tissues with high RFI steers having a higher cholesterol level that low RFI steers [176].

Altered lipid metabolism in the liver has previously been associated with RFI [178-180]. Using microarray analysis it was observed that key genes in lipid metabolism were elevated in high-RFI animals [180]. In a separate study, using RNA-seq technology, the authors hypothesised that low feed efficient animals had an increased lipogenesis in the liver [179]. This cohort of animals had greater fat deposition, elevated levels of serum cholesterol and up-regulated expression of *FASN* [179]. Similarly, an RNA-seq study in muscle tissue observed lipid metabolism to be altered between feed efficient phenotypes [181].

More recently a large cross-tissue RNA-Seq study aiming to identify gene networks associated with RFI, concluded that there was a deactivation of the regulatory network controlling FA metabolism in AT in the low RFI animals [182]. Similar conclusions were drawn relating to lipid metabolism and low RFI pigs [135].

1.5.6 Mitochondria

Mitochondria (Figure 1.4) often described as the "powerhouse of the cell" [183] are membrane bound, energy transducing organelles located in the cytosol of eukaryotic cells. Mitochondria drive cellular [184] metabolism by converting biological "fuels" into ATP through oxidative phosphorylation [185]. They are double membrane organelles. Both outer and inner membranes are phospholipid bilayers that contain proteins, The smooth outer membrane separates the mitochondria from the cytosol. The IMM is invaginated having folds called cristae which protrude into and define the matrix of the organelle [186]. The IMM is a highly complex system and is the site of oxidative phosphorylation. The cristae increase the surface area in which this

process can take place. Mitochondria have their own genome containing mitochondrial DNA (mtDNA), and they also divide independently of the cell in which they reside, indicating mitochondria replication is not coupled to cell division [186].

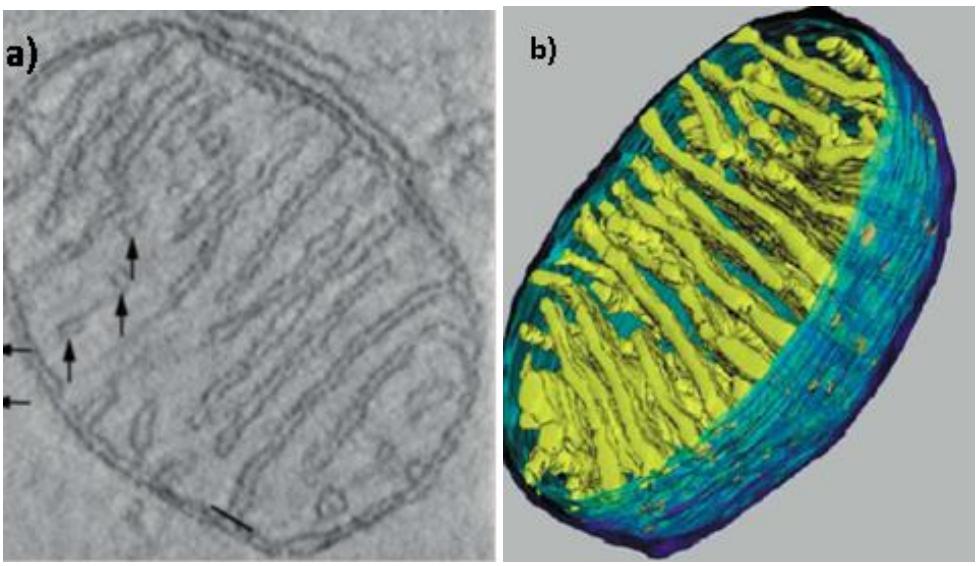

Figure 1.4: Mitochondrial structure.

(a) An electron micrograph of a mitochondria from chick cerebellum tissue and (b) A 3-dimensional model of a mitochondrian based on electron tomography [185].

1.5.6.1 Functions of mitochondria

Mitochondria are a highly dynamic organelle. Beyond a fundamental role in energy (ATP) production, they also play important roles in thermogenesis, sustaining cellular redox potential, calcium homeostasis [187], reactive oxygen species (ROS) production, fatty acid metabolism, pyrimidine biosynthesis, cell signalling [183] and cell death (apoptosis). With this in mind it is not surprising that mitochondrial dysfunction has been implicated in many rare genetic disorders, diseases of aging and defects in overall efficiency. The role of mitochondria in the above functions, while important, is beyond the scope of this review. The review will focus mainly on energy metabolism.

1.5.6.2 Energy Metabolism

Mitochondria have always been regarded as key organelles in the regulation of metabolism. Glucose, FFAs and glutamine are major energy sources in mammalian cells [188]. Many enzymatic reactions are vital to the complete oxidation of these molecules in mitochondria. Mitochondria are the sites or at least one of the sites at which many of these interconnected processes occur, including, β-oxidation of FFAs, ketogenesis, glutaminolysis, branched amino acid catabolism and the Krebs cycle. Figure (1.5) taken from Demine et al. (2014) illustrates the connections between these pathways [189].

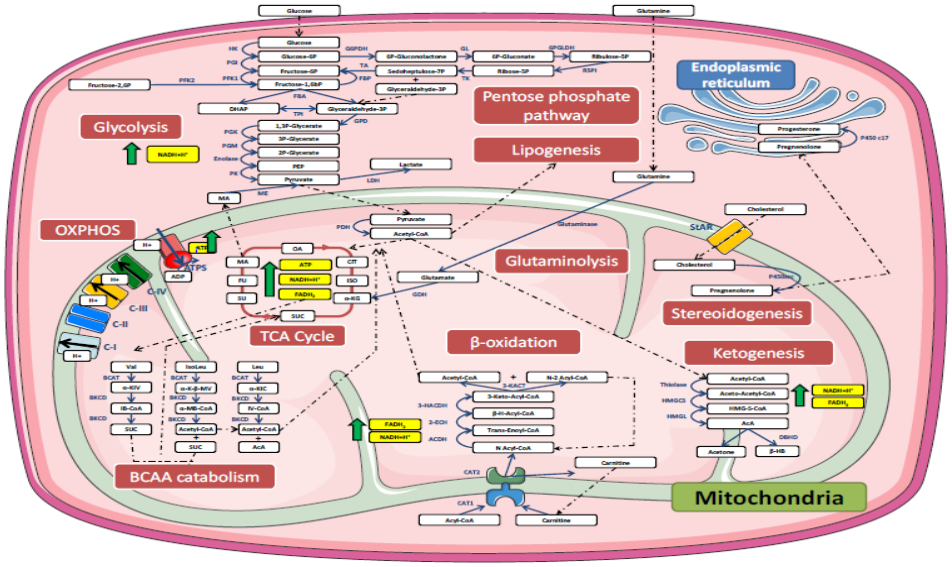

Figure 1.5. Illustration of the connected network of mitochondrial metabolic pathways.

See text (section 1.5.6.2) for more detail. Figure taken from Demine et al. (2014) [189].

β-oxidation of FFAs begins with conversion of FFAs into acyl-CoA esters by acyl-CoA synthase in the cytosol, followed by transportation of acyl-CoA into the mitochondria by the carnitine acyl-carnitine translocase system [190]. Once this translocation takes place, the steps of β-oxidation commence. These steps include dehydrogenation, hydration, the second step of dehydrogenation, thiolysis and production of a shorter acyl-CoA [189].

During glycolysis, a cytosolic process, glucose is metabolized through a series of reactions to produce pyruvate. Pyruvate is then metabolised further in one of two ways; a) in aerobic conditions pyruvate will be transported into the mitochondria where it will be converted by pyruvate dehydrogenase into acetyl-CoA or b) under anaerobic conditions it will be converted to lactate by lactate dehydrogenase. While the main function of glycolysis is to drive the Krebs cycle and ultimately produce ATP, it has been postulated that glycolysis provides "building blocks" for the synthesis of other metabolites. It has been observed that intermediates from glucose metabolism can be further processed to synthesize nucleotides, complex lipids and some amino acids. The reader is directed elsewhere for a more in depth discussion of these pathways [191].

The acetyl-CoA molecules formed are used by the Krebs cycle [191], or in the formation of lipids such as cholesterol among others [192]. In the Krebs cycle the acetyl-CoA is oxidized. The enzymes involved in the Krebs cycle are situated in the mitochondrial matrix except for succinate dehydrogenase. During the Krebs cycle the electron donors NADH and flavin adenine dinucleotide ($FADH_{2)}$ are produced. The electron transport chain (the oxidative phosphorylation system) and the Krebs cycle are conveniently located in close proximity to each other. This allows for the quick and efficient use of three molecules of NADH and one molecule of $FADH_2$ formed per one cycle of the Krebs cycle in the electron transport chain to produce ATP [189].

1.5.6.3 The electron transport chain

The electron transport chain (see Figure 1.6) also called the mitochondrial respiratory chain (RC) is the site of oxidative phosphorylation. It is embedded in the IMM and consists of four enzymatic complexes (CI-IV) and two electron carriers- ubiquinone and cytochrome c. The RC catalyses the transfer of reducing equivalents from high energy molecules produced during the reactions of the Krebs cycle to oxygen thus creating an electrochemical gradient called the proton motive force. This proton motive force works across the IMM to drive the synthesis of ATP by ATP synthase [98]. ATP synthase is commonly referred to as Complex V (CV) of the RC.

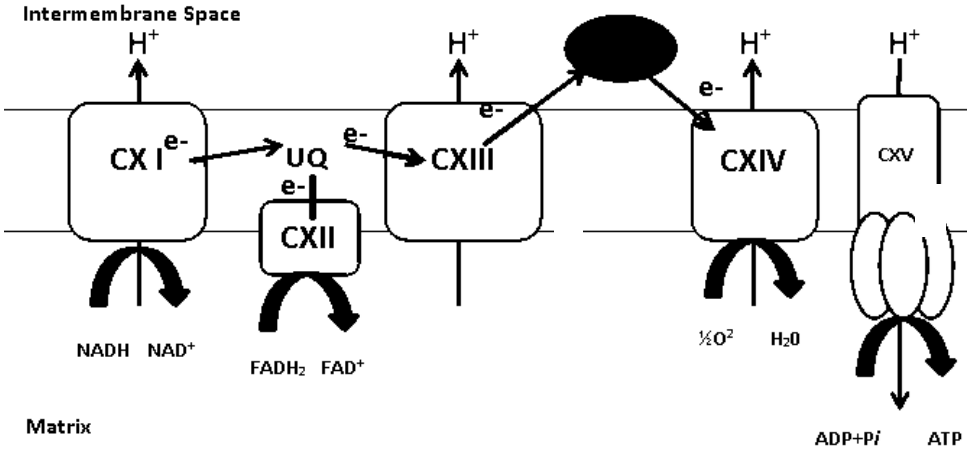

Figure 1.6. The electron transport chain embedded in the inner mitochondrial membrane.

The electron transport chain shows NADH-linked substrates with electrons (e-) entering the chain at complex I (CI). Electrons flow from CI and complex III (CIII) and Complex II (CII) and CIII by the action of coenzyme q/ubiquinone (UQ). Cytochrome c (cyt c) shuttles electrons from CIII and complex IV (CIV). The flow of electrons down the electron transport chain is accompanied by pumping of protons (H+), across the intermembrane space, creating a proton motive force that drives ATP synthesis when the protons move back through ATP synthase (CV). Illustration is authors own.

1.5.6.4 Reactive oxygen species

The electron transport chain (ETC) is a major site of ROS production and oxidative stress. It has been estimated that 2-4% of the total 0_2 consumed by mitochondria is converted to ROS by reduction of 0_2 to form superoxide as a result of electron leakage from the electron transport chain [193-195]. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide H_20_2, which is subsequently converted to H_20 by glutathione peroxidase. H_20_2 can be converted to the extremely reactive hydroxyl radical ($^{\cdot}OH$) in the presence of Fe^{2+} and Cu^{2+}. CI and CIII of the ETC are well known sights of ROS production, and while low levels of ROS are needed for normal cell functions (signal transduction pathways), over production of ROS can be extremely damaging to cells and has been linked to many diseases in humans [196, 197]. ROS can act as second messengers and are capable of altering gene and protein expression and the action of mitochondria.

1.5.6.5 MtDNA damage

Mitochondrial stability and integrity is dependent on a balance of mitochondrial and nuclear DNA [198]. Mitochondrial DNA is more susceptible to mitochondrial ROS-mediated oxidation than nuclear DNA (nDNA) due to its close proximity to the ETC and lack of protective histones. This oxidation can be a cause of mitochondrial dysfunction by repression of mitochondrial transcription which in turn inhibits respiratory protein synthesis [199, 200]. Reactive thiols in CI, CII and CIV of the respiratory chain are very susceptible to oxidation and damage of these thiols could decrease activity of the ETC [201].

1.5.6.6 Proton Leak, Uncoupling & Oxidative stress

As previously discussed, a proton motive force that develops from proton pumping across the IMM is used to drive ATP synthesis. Proton leak occurs when protons flow back into the mitochondria rather than into the ATP synthase. This action, known as uncoupling, dissipates the proton motive force, and it is estimated that proton leak can contribute as much as 25% of

total basal metabolic rate of an animal [202, 203]. While uncoupling can be considered a cellular inefficiency, it also reduces oxidative stress by mitigating mitochondrial ROS production. Uncoupling protein activity mediates this process [204-206].

1.5.6.7 Mitochondrial Biogenesis

During cell division, mitochondria divide such that daughter cells obtain a full complement of functional mitochondria. Despites this, in times of high energy demand, cellular signals are activated to increase mitochondrial number via mitochondrial biogenesis. These include Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), termed the master regulator of mitochondrial protein synthesis, Nuclear respiratory factor one (NRF-1) and NRF-2, and mitochondrial transcription factor A (mtTFA) [207, 208].

5' AMP-activated protein kinase is a vital regulator of animal food intake and overall energy balance, it is also a critical cellular energy sensor with the ability to stimulate mitochondrial biogenesis [209-213]. Once activated by phosphorylation, AMPK phosphorylates proteins involved in carbohydrate, lipid and protein metabolism [211, 212, 214]. In broad terms, AMPK increases energy levels by reducing ATP-utilizing processes such as FA synthesis and increases ATP-generating processes such as FA oxidation and glycolysis [197]. AMPK has been shown to upregulate PGC-1α expression [215], indicating an important role in mitochondrial biogenesis. Silent mating type information regulation 2 homolog (SIRT1) which is an important metabolic regulatory, is increased in response to AMPK, and together with PGC-1α these molecules regulate energy homeostasis in response to differences in nutritional and environmental factors [212, 216].

1.5.7 Feed efficiency and Mitochondrial Function

It is widely accepted that due to their significant role in energy metabolism, differences in function at the mitochondrial level could contribute largely to variation in FE. A study in breast

and leg muscle obtained from broilers demonstrated higher respiratory control ratio (RCR) values (a measure of respiratory chain coupling) in animals with superior FE phenotypes when provided pyruvate and malate as substrates. However, when succinate was introduced as a substrate there were no differences in RCR values between phenotypes. This observation led the authors to conclude that a site-specific defect was present in CI of mitochondria in muscle from inferior feed efficient broilers [217]. In the same study there were no differences observed in the adenosine diphosphate : Oxygen (ADP : O) ratio, indicating that birds with superior and inferior FE phenotypes had the same capacity for oxidative phosphorylation in muscle tissue.

Similar studies have examined mitochondrial function in the duodenum of broilers divergent in FE [218, 219]. Higher RCR's observed in the more feed efficient broilers indicated a possible defect of the ETC coupling at CII in mitochondria from the less efficient broilers.

In steers divergent for phenotypic RFI, a higher degree of coupling of the ETC (assessed either by RCR or ACR) in mitochondria from the *longissimus dorsi* muscle was observed in the low RFI steers [220]. Furthermore, in a separate study higher RCR values were reported in liver mitochondria from low RFI steers compared to their high RFI counterparts [221]. As in broilers, these studies reported no differences between low and high steers in their capacity to carry out oxidative phosphorylation.

A study designed to evaluate the relationship between hepatic mitochondrial function and RFI in growing beef cattle demonstrated that heifers with a low RFI tended to have a greater respiration rate and RCR values than heifers with a high RFI [221]. Despite this, there appeared to be no differences in respiration rate or RCR values between high and low RFI steers. The authors concluded that liver mitochondria in the low RFI animals had a greater control over oxidative phosphorylation.

Collectively these cross species studies indicate, that respiratory chain coupling and control in the liver was indeed better in more efficient animals compared to less efficient animals and that mitochondria from animals with a low feed efficient phenotype had the same ability to carry out oxidative phosphorylation as their high efficient counterparts [217]. In contrast to this [217, 220] reported similar ACR values but greater RCR values in muscle mitochondria of high feed efficient broilers and steers. Furthermore, a positive association between RCR and feed efficiency in muscle mitochondria has been reported in rats [222].

1.5.7.1 Feed efficiency and ROS production

Measuring site specific defects in electron transport and ROS production is a powerful way to examine problems with the ETC [223, 224]. With the exception of leg muscle, a large cross-tissue study (breast muscle, liver and duodenum) reported consistently higher basal H_20_2 levels in the less efficient broilers [184]. These experiments highlighted dysfunction in electron transport within CI and CIII in muscle mitochondria and CII in duodenal mitochondria in the less efficient broilers [217, 218]. It has been hypothesised that this increase in H_20_2 levels and subsequent ROS production is responsible for increased protein oxidation observed in the low feed efficient phenotype.

Ojano-Dirain et al. (2007) reported that uncoupling of the ETC reduced H202 production in low feed efficient animals but not in high feed efficient animals [225], indicating the low feed efficient phenotype have a lower mitochondrial membrane potential [197]. Previous studies have reported 10 to 30% greater heat production in cattle with high RFI compared to those with low RFI [226], in addition a positive correlation between maintenance energy requirement and RFI in growing steers has been established [93]. It has been hypothesised [221] that these differences in heat production between RFI phenotypes could be due to differences in the rate of proton-leak in muscle mitochondria (greater RCR in animals with low feed efficiency) [217, 220].

1.5.7.2 Feed efficiency and mitochondrial biochemistry

Studies in such tissues as muscle, duodenum and liver in poultry [227-229] sheep [230] and trout [231] have provided evidence of a relationship between FE and mitochondrial respiratory chain complex activities. With the exception of trout, these studies demonstrate compromised complex activity in the low feed efficient phenotype. In addition, a positive correlation was observed between reduced gluthathione (GSH), an antioxidant, and activities of CII, IV and V [219], suggesting that ROS-mediated damage to the protein complexes is at play in this scenario [197].

In direct contrast to the studies mentioned above, activities of all respiratory chain complexes were higher in low compared to high FE steers [232]. Despite this, increased oxidative stress and elevation of protein carbonyls (oxidatively damaged) in less efficient animals has been documented [228], indicating ROS-mediated oxidation may be responsible for lower complex activities and may in fact be contributing largely to variation in phenotypic FE [197, 228].

1.5.7.3 Feed efficiency and mitochondrial protein expression

As previously mentioned increased ROS can affect gene and protein expression [214, 233-235]. Studies in broilers divergent for feed efficiency, demonstrate differential protein expression of cytochrome oxidase subunit II (COX II) of CIV and cytochrome c1 of CIII in several tissue types [219, 236, 237]. Liver tissue and lymphocytes from feed efficient broilers appear to have a higher protein expression of the ATP synthase–α subunit of CV [236, 237] while (Adenine Nucleotide Translocator 1) ANT-1 (responsible for ADP/ATP transport) protein expression was higher in muscle tissue from less feed efficient broilers [238]. In contrast to this, in muscle obtained from steers, the low RFI animals had a greater expression of ANT [239].

Lassiter et al. (2006) observed differential expression of 5 key proteins involved in the ETC in lymphocytes[237]. Expression of Core I and cyctochrome c 1 subunits of CIII and ATP synthase –α of CV were higher in lymphocytes more efficient broilers, while the 30 subunit of

CII and COX II subunit of CIV were higher in lymphocytes obtained from the low efficient broilers.

In cattle, Complex 1 protein concentration in mitochondria isolated from lymphocytes was greater for low RFI animals compared with high RFI animals, in addition subunit S1 of the CI protein was higher in low RFI cattle [240]. A more recent study by the same group [241] reported no differences in protein concentrations of Complexes in lymphocytes from cattle divergent in RFI. The mitochondria complex protein concentration ratio of CI: CII increased 20% and the ratio of CI: CIII increased 30% in low RFI steers when compared with their high RFI counterparts. These authors hypothesised that a greater ratio of CI proteins in relation to CIII would facilitate an increase in shuttling of NADH into the mitochondria and subsequently a faster return to phosphorylation homeostasis [241].

A study was conducted to attempt to identify differentially expressed proteins (mitochondrial and extramitochondrial) in broilers divergent for feed efficiency, using a proteomics approach. Proteins identified as being differentially expressed between phenotypes include collapsin-2, vinculin (cell to cell communication), plasma transthyretin (thyroid hormone and retinol transport), histone deacetylase (gene regulation), and adophilin (lipid accumulation) [237].

Modifications in mitochondrial metabolism were reported at protein level in adipose tissue of pigs divergent in RFI [135]. In low RFI pigs, an overexpression of the mitochondrial multienzyme complex pyruvate dehydrogenase, which catalyses the conversion of pyruvate to acetyl-CoA, and dihydrolipamide dehydrogenase which is involved in pyruvate dehydrogenase activity was reported. Proteins that have antioxidant functions and that are involved in the oxidation-reduction processes and stress resistance were expressed at higher levels in the low RFI pigs [135]. These results are all consistent with the hypothesis that less efficient animals are subject to more oxidative stress and that more efficient animals have a better capacity to protect against oxidative stress.

Most recently, a shotgun proteomics experiment [242] in muscle tissue from broilers divergent in FE, listed mitochondrial function and oxidative phosphorylation as the first and fifth top canonical pathways in the proteomics dataset. In addition to this the same authors observed a skewing of the mitoproteome towards high feed efficient birds. The authors used the data to predict that there was an increase in respiratory chain activities in the high feed efficient birds. Furthermore, ANT and voltage-dependent anion channel (VDAC2), two important mitochondrial proteins were observed to be up regulated in high FE birds. The combined activities of these two proteins are responsible for shuttling ADP and ATP between the mitochondrial matrix and the cytosol thus facilitating cellular work while simultaneously providing substrate for ATP synthase that uses proton motive force generated by the respiratory chain to produce ATP from ADP [242].

Finally, Peroxiredoxin III (PRDX3), an antioxidant-like protein, which is responsible for maintaining mitochondrial mass and membrane potential, was predicted to be up regulated in muscle from high FE birds [242]. It is evident from the literature that there is deviances in protein expression between animals that are divergent for FE, however further investigation to discern the exact role of mitochondrial protein expression is warranted.

1.5.8 Blood metabolites

Blood metabolites can be used and as indicators of metabolic status in animals divergent in RFI [243, 244]. The use of blood metabolites and hormones as physiological markers of FE in beef cattle has been previously considered [245-247]. Plasma Insulin-like growth factor (IGF-I) has been suggested as a possible marker for assisting selection for low RFI [246]. However subsequent studies investigating the suitability of plasma IGF-I as a marker have found that it may not be a suitable indicator of RFI in beef cattle. Lancaster et al. (2008) found that selection for post weaning IGF-I had little to no effect on the selection for low RFI cattle as the relationship between RFI and plasma IGF-I concentration may be influenced by composition

of growth and age at blood sampling [248]. Similarly, in multiple studies no relationship was found between plasma concentrations of IGF-1 and phenotypic RFI in growing beef cattle [26, 244, 247]. Despite this Welch et al. (2013) suggested that the growth hormone (GH)-IGF axis may have some involvement with RFI at the molecular level [249].

The hormone insulin contributes significantly to many physiological processes such as growth, development and nutrient homeostasis [76, 244, 250]. It follows that some of the differences in RFI between animals could be attributed to differences in plasma concentrations of insulin. The literature regarding RFI and Insulin is conflicting. Studies have found a positive correlation [65, 247, 251] a negative correlation [252] and no relationship [13, 26, 60, 220] between RFI and plasma Insulin. It is possible that variation in plasma insulin concentration is indicative of skeletal muscle insulin sensitivity or resistance [112] which has implications for glucoregulatory mechanisms such as gluconeogenesis and glycolysis.

Energy status of beef animals can be estimated using indicators such as the plasma concentration of certain blood metabolites, such as glucose, non-esterified fatty acids (NEFA) and β-hyrdoxy butyrate(βHB) [253]. Plasma glucose levels have been observed to have no relationship with RFI [26, 58, 60, 252, 254] and a positive relationship [255]. When ruminants are fed below maintenance and hepatic ketogenesis and fat mobilisation occur they exhibit an increase in production of ketone bodies, such as βHB and NEFA [112]. The literature contains conflicting reports regarding the relationship of NEFA and RFI. Many authors have found no relationship between RFI and NEFA [252, 254, 256], however, others [26, 60] found higher concentrations of NEFA in low RFI beef animals agreeing with the study of [257]. This conflict in the literature is applicable to the relationship between βHB and RFI also. Some authors [60, 252, 255, 257, 258] observed no difference, whereas others [26, 254], found that βHB levels were higher in high, compared to low RFI beef heifers.

Creatine phosphate, a marker for muscle mass, is an energy storage compound found in muscle, when it becomes degraded circulating blood creatinine levels increase [259]. Many studies [79, 258] found no relationship between plasma creatinine levels, but in contrast, others [60, 244, 257] found that low RFI animals had an increased circulating concentration of creatinine which is indicative of a greater muscle mass in feed efficient animals.

1.5.9 Activity

A variation in heat production, and subsequent availability of energy for maintenance and growth, can occur as a result of differences in energy expenditure associated with activity of the animal [20]. Variation in activity of an animal has the potential to contribute significantly to variation in RFI. For example, in pigs, it has been documented that total daily feeding time and numbers of visits to a feeding station are positively correlated with RFI [260]. In addition, in poultry, variation in activity contributes to a substantial proportion of the variation in RFI in chickens [261, 262]. Mice, divergently selected for heat loss, were shown to have different levels of activity, with the high heat loss (low efficiency) mice being nearly twice as active as their high efficiency counterparts [263]. These authors concluded that this difference in activity accounted for 36% of the difference in feed intake between selected lines. These results were further validated by L Bunger et al. (1998) who observed that high feed intake mice (low efficiency) were 3 times more active than their more efficient counterparts [264].

In cattle, research has demonstrated that about 10% of the observed variation in RFI can be attributed to variation in activity between the phenotypes. Richardson et al. (1999) reported a phenotypic correlation of 0.32 for RFI with daily pedometer count. Physiological mechanisms affected by variation in activity include work involved in feeding, ruminating and locomotion at various speeds [20].

1.5.10 Thermoregulation

Evaporative heat loss, through heat exchange in the lungs and nasal turbinates, is the primary route of energy loss in ruminants [265]. This process is largely regulated by rate of respiration. Changing posture, wetting, seeking shelter and huddling do not contribute hugely to variation in heat loss, with the exception of extreme situations [63].

It has been observed that hens with a lower RFI value have smaller nude areas on their body from which they could lose heat, slightly better feather coverage and were less active than their high RFI counterparts. Each of these factors is likely to affect thermoregulation and is possibly a contributing factor to variation in RFI in poultry [262]. However, the large difference in body size of cattle and chickens may cause these factors to be different in cattle.

1.6 GENOMICS

All the genetic information possessed by a living organism is referred to as the genome. The DNA, which contains the coding and non-coding sequences is arranged into chromosomes and all the genetic information contained here constitutes an individual's genome [266]. The genome of the Hereford cow, L1 Dominette 01449 was initially generated and assembled by the Baylor College of Medicine Human Genome Sequencing Centre, and the *Bos Taurus* genome assembly went through several stages of improvement [267]. The Bovine Genome Sequencing and Analysis Consortium published a paper in Science in 2009 in which they detailed their work to annotate and analyse assemblies Btau_3.1 and Btau_4.0 [268]. Simultaneously, the University of Maryland used the same genomic sequence data to generate an alternative assembly, named UMD2, which was published in Genome Biology in 2009 [269]. Both versions have been updated to their current versions, Btau_4.6.1 and UMD3.1.1. It is widely understood that the existence of two competing genome assemblies is not an ideal situation for the bovine research community and different researchers may find advantages with

one over the other, for example, Btau_ 4.6.1 includes the Y chromosome while UMD3.1.1. does not. In contrast, while UMD3.1.1. contains the mitochondrial genome, Btau_4.6.1 does not [270]. The genome contains approximately 22,000 protein-coding genes of which 14,345 are shared among 7 mammalian species [268].

Genomics refers to the study of genes and their functions and employs molecular biology techniques to identify genes, their expression levels and to elucidate their biological function [271]. Genomics is divided into structural and functional genomics. Structural genomics characterises the physical nature of the genome whilst functional genomics characterises the overall patterns on gene expression [271, 272].

1.6.1 Functional Genomics

Functional genomics characterises overall patterns of gene expression [271, 272] and detects genes that are switched on and off at certain time points depending on environmental factors [271]. Functional genomics can quantify gene expression through the use of techniques such as transcriptomics, proteomics and metabolomics.

Real time quantitative polymerase chain reaction (RT-qPCR) is a rapid and accurate transcriptomic technique that is used to measure target gene expression in samples. This method can be applied to model systems to measure responses to experimental stimuli and to gain insight into potential changes in protein level and function [273]. Indeed, there is a plethora of studies in which RT-qPCR has been used to investigate potential biological pathways and mechanisms involved in variation in RFI. The technique has been utilised in studies to examine the relationship between RFI and expression of genes relating to energy metabolism [247, 274].

RNA sequencing (RNA-seq) enables rapid profiling and deep investigation of the transcriptome detecting novel or known features and quantifying RNA activity [275]. RNA-seq has many advantages over previously used techniques such as RT-qPCR and microarrays as it does not

require species or transcript-specific probes. It can detect novel transcripts, gene fusions, single nucleotide variants, indels (small insertions and deletions), and other previously unknown changes that arrays cannot detect [108].

Using these functional genomic techniques to examine differential gene expression may not only explain some of the observed variation in RFI but these genes may also be potential biomarkers or may be used to identify SNPs, for genomic assisted selection, as described in the next section.

1.6.2 Genomic Assisted Selection for RFI

It is well established that feed efficiency has a strong underlying genetic basis, and is, for the majority of cases, not antagonistically correlated with other economically relevant traits [22, 52]. Genetic selection to reduce RFI can result in progeny that consume less without compromising on growth performance [243] indicating that it can be passed on to future generations, making it a favourable measure of efficiency. However, the main challenges to widespread adoption of RFI are the expense and logistics associated with measuring the trait [4]. The cost of generating sufficient RFI data by developing well established populations of animals, and also the technical difficulty in measuring the trait has caused producers to be reluctant about including FE as an economically relevant trait in breeding programmes. RFI calculation in cattle requires individual animal identification and specialised equipment for the accurate measurement of feed intake and liveweight over a minimum period of 70 days. These costs are even further increased when measuring cattle on pasture based diets due to costs associated with provision of suitable forage. Testing one animal for feed intake has been estimated at €140 [49] leading research in FE to be directed towards understanding the molecular basis for FE. Utilising predictor traits or biomarkers that are logistically easy and inexpensive to measure would reduce cost and expedite selection for feed efficient animals. Traditionally, genetic improvement of livestock used phenotypic information and pedigrees to

predict breeding values. More recently, the prediction accuracy of these breeding values may be enhanced by using information on variation in DNA sequences between animals, known as marker-assisted selection [276]. This technique uses genetic markers that are linked to regions of the DNA that are of interest or relevance to producers. Genomic selection uses a large panel of dense, genomic-wide markers known as SNPs to predict breeding values of animals [277, 278]. RFI is considered as a prime candidate for genomic selection due to its moderate heritability [22] and genetic variation [4]. Many studies have attempted to elucidate the genetic background behind this complex trait across many species, using approaches such as linkage analyses, genome-wide association studies (GWAS), candidate gene association and transcriptomic profiling. For example, GWAS and linkage studies [279-282], candidate gene approaches [283-285] and transcriptomics [179, 182, 286-289] have been performed. These previous studies have revealed potential candidate genes and provide important background information for genetic control of RFI.

1.6.3 Feed efficiency and Gene Expression

When studying protein and gene expression it is important to consider that mRNA levels and protein expression do not always coincide, due in part to mRNA stability and posttranslational modification of proteins [290].

Using RT-qPCR, mRNA levels of ANT were observed to be lower in muscle from more feed efficient broilers [238]. In contrast to this Kelly et al. (2011) observed a higher expression of ANT in more efficient cattle [247]. Less efficient broilers [238] and cattle [247] exhibited lower mRNA levels of COX III (possibly due to oxidative damage). The mRNA expression of UCP3 and PGC1α were altered in muscle tissue between cattle of divergent in RFI [247].

Using microarray technology, Chen et al. (2011) found that 161 unique genes were differentially expressed between cattle of high and low RFI [286]. The differentially expressed

genes (DEG) were involved in several gene networks involved in cell growth and proliferation, protein synthesis and lipid and carbohydrate metabolism, which would suggest that vital cellular mechanisms could be contributing to variation in RFI in beef cattle.

More recently, RNA-seq technology has become the favoured technique when investigating the mechanisms contributing to the molecular control of FE. Genes involved in ion transport and binding, membrane binding, transport and catalysis of molecules through the cell membrane and essential mechanisms of nutrient absorption were found to be differentially expressed in liver from Nellore steers divergent in RFI [287]. Cattle with low FE presented a hepatic transcriptome associated with pronounced inflammation and lipid metabolism [179]. A large, cross tissue RNA-seq study provided a list of DEG and gene co-expression networks linking DEG with tissue function, and transcription factors [182]. The authors hypothesised that genetic predisposition for FE is associated with subtle changes in a variety of processes [182]. Most recently, a study reported differences in genes involved in metabolism of Xenobiotics by Cytochrome P450, Butanoate and Tryptophan metabolism, mitochondrial function and the metabolism of lipids in muscle of cattle divergent for RFI. The findings indicate that observed gene expression differences are primarily related to metabolic processes underlying oxidative stress [181].

1.7 Objectives of this study

The aims of this thesis were to (i) increase our understanding of the underlying biology governing FE, as measured by RFI in a variety of metabolically active tissues, (ii) identify novel molecular mechanisms explaining differences in feed efficiency in beef cattle and (iii) to examine the transcriptome of animals divergent for RFI across multiple breeds and diets.

Chapter 2

CHAPTER 2. MATERIALS AND METHODS

2.1 Animal Models

2.1.1 Project licences

All procedures involving animals in Chapters 3, 4 and 5 were conducted under an experimental license (AE19132/P011) from the Health Products Regulatory Authority in accordance with the cruelty to Animals Act 1876 and the European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005.

All procedures involving animals in Chapter 6 were conducted under an experimental license (AE19132/P029) from the Health Products Regulatory Authority in accordance with the cruelty to Animals Act 1876 and the European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005.

2.1.2 Chapter 3-5

2.1.2.1 Animal management

The animals used in Chapters 3-5 were derived from a purebred herd of Simmental cattle originally established to examine various aspects of the biological control of the RFI trait and which has been well characterised to date in the published literature [27, 60, 72, 111, 254, 256, 291]. In order to generate animals for the current study, the 20 highest (inefficient) and 20 lowest (efficient) ranking cows and heifers on RFI within the herd were subjected to a multiple ovulation and embryo recovery programme and breeding using artificial insemination to bulls with high and low economic breeding values for RFI, respectively [22]. Resultant embryos were transferred to crossbred beef heifer recipients. Pregnant heifers were managed under standard protocols and following calving, were allowed to suckle their calves for a period of up to 7 days. In order to standardise rearing, calves were then abruptly weaned and were subsequently reared on an electronic calf feeder. Briefly, calves were offered milk replacer

(MR) (Blossom Easymix; Volac, Co. Cavan, Ireland) and concentrate in pelleted form using an electronic feeding system (Vario; Foster-Tecknik, Engen, Germany), which recorded all feed-related events including intake of both MR and concentrate, drinking speed, as well as number of rewarded (when calves receive milk) and unrewarded (no milk dispensed) visits to the machine. Calves were subsequently weaned at 10 weeks of age and were offered concentrate and hay on a 50:50 dry matter basis until turnout to pasture, at approximately six months of age. At approximately 15 months of age all cattle were housed within pens of between 5-7 animals/pen in a slatted floor shed. Cattle were fed once daily (0800 h) and were offered *ad libitum* concentrate (860 g/kg rolled barley, 60 g/kg soya bean meal, 60 g/kg molasses and 20 g/kg minerals/vitamins) and 3kg grass silage to retain ruminal function. The animals had an acclimatisation period of 14 d to the *ad libitum* regime and test facilities before the experimental recoding period commenced. Feed intake was recorded daily and body weight (BW) was recorded twice weekly. The recording period lasted 70d.

2.1.2.2 Feed analysis

Concentrate and silage offered were sampled three times weekly and samples were stored at -20°C pending laboratory analysis. Samples of concentrates and silage were subsequently pooled on a weekly basis for dry matter (DM) determination. Concentrate samples were dried in an oven with forced-air circulation at 98°C for 16 h for DM determination and forage samples dried at 40°C for 48 h.

2.1.2.3 Body weight and body measurements

Animals were weighed prior to feeding, at the start and at the end and on a weekly basis throughout the RFI measurement period. All animals were ultrasonically scanned by the same person at the beginning and end of the RFI measurement period using a dynamic real-time scanner (Honda HS 2000 with 2 MHz transducer probe, Honda Electronics Co., Ltd.,

Toyohashi, Japan) to measure fat depth at the third lumbar vertebra, the 13th thoracic rib and the rump (P8 site) on the animal's right side [292].

2.1.3 Chapter 6

This experiment was conducted as part of larger study designed to examine the within-animal repeatability of intake, growth and feed efficiency between the growing and finishing stages of the lifespan of Charolais (CH) and Holstein Friesan (HF) beef steers offered either the same diet or diets contrasting in energy density and chemical composition [62]. Briefly, one hundred and sixty seven cattle comprised of 90 CH and 77 HF were used in the study. For the purpose of this study, feeding phases 1, 3 and 4, in which cattle were offered high concentrate in the growing phase, zero grazed grass in the growing phase and high concentrate again at the finishing stage were examined. Following a dietary adaptation period, individual dry matter intake (DMI) and growth were measured over three 70 d feeding phases. Mean BW (SD) and age (SD) at the start of the first feeding phase were 395 kg (37.8) and 283 d (18.3), and 294 kg (42.3) and 306 d (7.7), for CH and HF, respectively. Corresponding BW at the start of phase 3 and phase 4 were 519 (38.3) and 441 (39.2), and 675 (49.8) and 611 (49.3), respectively. During feeding phases 1 and 3 (high concentrate feeding phases 1 and 2, respectively) steers were individually offered the same concentrates (860 g/kg rolled barley, 60 g/kg soya bean meal, 60 g/kg molasses, and 20 g/kg minerals and vitamins) *ad libitum* plus a restricted allowance of grass silage daily. Throughout the interim period they were offered zero-grazed grass (ZGG; DM 196 g/kg) *ad libitum*. The grass herbage was harvested (without chopping) twice daily from *Lolium perenne* dominant swards using a 'zero-grazer'.

2.2 Computation of traits and RFI calculation

2.2.1 Chapters 3-5

Heifers and bulls were considered as separate groups for computation of traits and statistical analysis. Average daily body weight gain during the RFI measurement period for each animal was computed as the coefficient of the linear regression of body weight (BW) (kg) on time (d) using the GLM procedure of SAS 9.1 (SAS Inst. INC., Cary, NC). Mid-test metabolic BW (MBW) was represented as $BW^{0.75}$ 35 d before the end of the test, which was estimated from the intercept and slope of the regression line. Expected DMI was computed for each animal using a multiple regression model, regressing DMI on MBW, average daily gain (ADG) and mean lumbar backfat (BF) change. Residual feed intake was calculated within gender for each animal as the difference between actual DMI and expected DMI.

The model used to compute expected DMI was

$$Yj = \beta 0 + \tau i + \beta 1 MBWj + \beta 2 ADGj + \beta 3\ BFj + ej$$

where Y*j* is the average of the *j*th animal, $\beta 0$ is the regression intercept, τi is the fixed effect of the *i*th year, $\beta 1$ is the partial regression coefficient for MBW, $\beta 2$ is the regression coefficient for ADG, $\beta 3$ is the regression coefficient for BF and e*j* is the random error associated with the *j*th animal. The coefficient of determination (R^2) for the statistical model produced from this equation accounted for 0.7 ($p<0.001$) of the variation in DMI and was used to predict DMI for each animal. Animals were ranked according to RFI within gender and then classified as low RFI (efficient), and high RFI (inefficient). For Chapter 3 this resulted in 8 animals per group in each of the high and low RFI heifer groups, and 9 animals per group in each of the high and low RFI bull groups, respectively. For Chapter 4 this resulted in low RFI (n=8 and n=9) and high RFI (n=8 and n=9) for bulls and heifers, respectively. Following a power analysis

(www.biomath.info/power/ttest.htm) the most extreme animals for high RFI (n=5) and low RFI (n=5) were selected in Chapter 5 for RNA-seq.

2.2.3 Chapter 6

The residuals of the regression of DMI on average daily gain (ADG), mid-test metabolic BW (MBW) within each breed, were used to compute individual RFI coefficients for each feeding phase using the GLM procedure of SAS 9.1 (SAS Inst. INC., Cary, NC). Residual feed intake was calculated for each animal as the difference between actual DMI and expected DMI. Animals were ranked within breed and feeding phase for high RFI (n=12) and low RFI (n=12).

2.3 Sample collection

2.3.1 Biopsy sample collection

M. longissimus dorsi biopsies were harvested for Chapters 3,5 and 6 as described by [247] and liver tissue was collected for Chapter 3 and 5 by percutaneous punch biopsy as described by [293] from animals deemed high and low RFI under local anaesthetic (5 mL Adrenacaine, Norbrook Laboratories (Ireland) Ltd.) at the end of the RFI measurement period. All surgical instruments used for tissue collection were sterilised and treated with 70% ethanol and RNaseZap (Ambion, Applera Ireland, Dublin, Ireland). *M. longissimus dorsi* biopsies were snap frozen in liquid nitrogen directly after collection and liver biopsies were washed in sterile DPBS and snap frozen in liquid nitrogen. All samples were subsequently stored at -80°C pending further processing.

2.3.2 Animal slaughter and tissue collection

All animals pertaining to Chapters 3-5 were slaughtered at an EU licenced abattoir (Eurofarms Foods, Duleek, Co. Meath, Ireland). Details of slaughter metrics are presented in Table 9.1 Animals were weighed prior to slaughter. The mean (SD) bodyweight and age for bulls and

heifers at slaughter were 660 kg (67 kg) and 18.8 months (1 month), and 576 kg (38 kg) and 18.5 months (0.9 months), respectively. All surgical instruments used for tissue collection were sterilized and treated with RNase Zap (Ambion, Applera Ireland, Dublin, Ireland). A sample of subcutaneous adipose tissue (SCAT) was collected immediately, from the rump area, after hide removal. Samples were washed with sterile PBS, cut into small pieces and snap frozen in liquid nitrogen and subsequently stored at -80°C until use in Chapter 4.

2.3.3 Blood metabolites

For the purpose of Chapter 3 blood samples were obtained by jugular venepuncture from each animal prior to feeding, on d 1 and 72 of the RFI measurement period. Blood samples were collected into a 9- and 4-ml evacuated tube containing lithium heparin and sodium fluoride – EDTA K_3, respectively, as anticoagulants (Greiner Vacuette, Cruinn Diagnostics, Dublin, Ireland).

2.4 Sample processing

2.4.1 Tissue homogenate preparation and protein determination

In Chapter 3 tissue homogenates of *M. longissimus dorsi* and liver biopsy samples were prepared as outlined by [294] with slight modifications. Briefly, visible fat and connective tissue was removed from 50 mg of tissue, which was then dissected into small fragments. Tissue was diluted at a ratio of 1:20 in ice- cold sucrose (250mM) homogenisation buffer (pH 7.4) containing; tris(hydroxymethyl)aminomethane (Tris; Sigma Aldrich Ireland Ltd., Wicklow, Ireland), potassium chloride (KCl; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) and ethylenebis (oxyethylenenitrilo)tetraacetic acid (EGTA; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) diluted in distilled water. Sucrose (Sigma Aldrich Ireland Ltd., Wicklow, Ireland) was added on day of use. Tissue was homogenised using a clean glass-glass conical tissue grinder on ice. Homogenate was centrifuged at 600*g* for 10 min at 4°C and supernatant was kept on ice

and used on the same day. Protein concentration of homogenates was determined using the Pierce BCA Protein Assay Kit (Fisher Scientific, Ireland).

2.4.2 RNA isolation and purification

In Chapters 4-6 total RNA was isolated from 50mg of muscle, liver and adipose tissue. QIAzol (Qiagen, UK). Tissue samples were homogenised in 3mL of QIAzol reagent using a rotor-strator tissue lyser (Qiagen, UK) and chloroform (Sigma-Aldrich Ireland, Dublin, Ireland). RNA was subsequently precipitated and purified using the RNeasy plus Universal kit (Qiagen, UK) according to the manufacturer's guidelines, which included a step to remove any contaminating genomic DNA. The quantity of the RNA isolated was determined by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). RNA quality was assessed on the Agilent Bioanalyser 2100 using the RNA 6000 Nano Lab Chip kit (Agilent Technologies Ireland Ltd., Dublin, Ireland). RNA quality was also verified by ensuring all RNA samples had an absorbance (A260/280) of between1.8 to 2.0 and RIN values of between 8 and 10 were deemed high quality. Any samples that had a (A260/280) absorbance of less than 1.8 were cleaned using a Zymo Research RNA clean & concentrator kit (Cambridge Biosciences, UK). High quality RNA samples were selected for subsequent cDNA synthesis.

2.4.3 Blood metabolite analysis

In Chapter 3 Concentrations of albumin, urea, globulin, total protein, β-hydroxybutyrate (βHB), glucose, non-esterified fatty acids (NEFA), triglycerides and creatinine were determined according to [254]. Globulin concentration was calculated as the difference between total protein and albumin concentrations. All metabolite concentrations were measured on an automatic analyser (AU 400; Olympus, Tokyo, Japan). The sensitivity of each assay was defined as the lowest concentration detectable.

2.5 Enzymatic assays

Citrate Synthase Assay

The activity of citrate synthase was assayed by coupling the rate-limiting reaction catalysed by citrate synthase (Eq1) to the irreversible chemical reaction (Eq2):

Acetyl-CoA + oxaloacetate + H_2O = citrate + CoA-SH (Eq1)

CoA-SH + 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) = TNB + CoA-S-S-TNB (Eq2)

The reaction product 5-thio-2-nitrobenzoic acid (TNB) has an intense absorption at 412 nm and the absorbance increases linearly with time. This increase in absorption was measured for 4 min and rate of reaction was calculated. The enzymatic activity of citrate synthase was calculated as nmol/min/mg mitochondrial protein using the molar extinction coefficient forcitrate synthase, which is 13.6 mM/cm [294].

Measurement of Mitochondrial Electron Transport Chain Complex Activities

The activity of electron transport chain complexes were assessed in muscle and liver biopsy samples by UV spectrophotometry as described by Spinazzi et al. (2012) with modifications using the Shimadzu UV-2600 spectrophotometer. All assays were performed in duplicate at 37°C in 2 mL cuvettes. Activities of all complexes were calculated as nmol/min/mg of protein and expressed in units normalised to citrate synthase activity which is a marker of mitochondrial abundance within a tissue/cell.

Complex I Activity. The activity of complex I (CI) was assayed as a decrease in absorbance at 340 nm by following the oxidation of reduced nicotinamide adenine dinucleotide (NADH; Sigma Aldrich Ireland Ltd., Wicklow, Ireland). Mitochondria (40 µg of tissue homonegnate) were incubated at 37°C for 2 min in 700 µL of reaction medium [50 mM Tris-Hydrogen Chloride (HCL; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) with 3 mg/mL Bovine Serum

Albumin (BSA; Fisher Scientific, Ireland), pH 8.0; 300 μM potassium cyanide (KCN; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) and 100 μM NADH]. 60 μM Ubiquinone (Coenzyme Q1) was added to initiate the reaction. Absorbance was monitored for 3 min before and after the addition of 10μM Rotenone (Sigma Aldrich Ireland Ltd., Wicklow, Ireland). The difference in the decrease in absorption due to NADH oxidation was measured in the absence and presence of rotenone and the rotenone-sensitive activity of CI was subsequently quantified using an extinction coefficient of 6.22 mM/cm [294].

Complex II Activity. Complex II (CII) activity was determined by following the secondary reduction of 2, 6-dichlorophenolindophenol (DCPIP; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) by decylubiquinol (DUB; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) at 600 nm. Mitochondria (2 ug of protein) were added to a buffer containing 25 mM Potassium dihydrogen phosphate (KH_2PO_4; Sigma Aldrich Ireland Ltd., Wicklow, Ireland), 0.10 mM EDTA (Sigma Aldrich Ireland Ltd., Wicklow, Ireland), BSA (1 mg/ml), 300 μM KCN, 80 μM DCPIP and 20mM succinate (Sigma Aldrich Ireland Ltd., Wicklow, Ireland) and incubated for 10 min. 50 μM DUB was added and the reaction was initiated with the addition. Absorbance was monitored for 3 min before and after the addition of 10 mM malonate (Sigma Aldrich Ireland Ltd., Wicklow, Ireland) which acts as an inhibitor of the reaction. The difference in the decrease in absorption before and after addition of Malonate represents the complex activity. The extinction coefficient used for this reaction was 19.1 mM/cm [294].

Complex III Activity. The assay was performed at 550 nm by monitoring the rate of reduction of cytochrome c by ubiquinol-2. Mitochondria (3 μg of protein) were added to medium containing 25 mM KH_2PO_4 (pH 7.8), 0.10 mM EDTA, and 75μM oxidized cytochrome c (Sigma Aldrich Ireland Ltd., Wicklow, Ireland) and left to incubate for 2 min. The reaction was initiated by addition of 100 μM decylubiquinol (Sigma Aldrich Ireland Ltd., Wicklow, Ireland) and followed for 2 min. The non-enzymatic reduction of cytochrome c was measured after

addition of 4 μM antimycin A (Sigma Aldrich Ireland Ltd., Wicklow, Ireland); the specific activity of complex III (CIII) was calculated by subtracting the non-enzymatic rate. An extinction coefficient of 18.5 mM/cm was used [294].

Complex IV activity. The activity of complex IV (CIV) was measured by following the oxidation of reduced cytochrome c (cytochrome c was reduced with sodium dithionite) as a decrease in absorbance at 550 nm. The reaction medium contained 25mM KH_2PO_4, 1 mg/mL defatted BSA, and mitochondria (25 to 40 μg of protein). The reaction was initiated by adding 60 μM reduced cytochrome c, and the reaction was followed for 60 s. The specific activity was calculated by using 18.5 mM/cm as the extinction coefficient [294].

Complex I + III combined activity. In order to measure the flow of electrons between complexes the combined activity of CI and CIII was measured by monitoring the reduction of oxidised cytochrome c at 550nm. Mitochondria (25 μg of protein) were added to a medium containing 50 mM KH_2PO_4, 1 mg/mL BSA, 300 μM KCN and 50 μM oxidised cytochrome c and left to incubate and the baseline for the reaction was recorded for 2 min. The reaction was initiated by the addition of 200 μM NADH. The differences in absorption were measured in the presence and absence of rotenone and the combined specific activity was recorded as the rotenone sensitive activity. The specific activity was calculated by using 18.5 mM/cm extinction coefficient [294].

Complex II + III combined activity. In order to measure the flow of electrons between complexes the combined activity of CII and CIII was measured by monitoring the reduction of oxidised cytochrome c at 550nm in the presence of succinate. Mitochondria (3 μg of protein) were added to medium containing 20 mM KH_2PO_4, 1 mg/mL BSA, 300 μM KCN and 10mM succinate. Samples were pre-incubated with succinate for 10 mins to fully activate the enzyme. The reaction was initiated by addition of 50 μM oxidised cytochrome c. The differences in absorption were measured in the presence and absence of malonate and the combined specific

activity was deemed as the malonate sensitive activity. The specific activity was calculated by using 18.5 mM/cm extinction coefficient [294].

2.6 Statistical Analyses

2.6.1 Effect of RFI grouping on performance traits, mitochondria and blood metabolites

In Chapter 3 normality of data distribution was tested using the UNIVARIATE procedure of SAS 9.1 (SAS Institute Inc.). Data that were not normally distributed were transformed by raising the variable to the power of lambda. The required lambda value was calculated by conducting a Box–Cox transformation analysis using TRANSREG procedure of SAS. Data subjected to transformation were used to calculate *P*-values. The least squares procedure of SAS was used to examine the effect of RFI grouping on feed intake, performance traits, body composition, mitochondrial complex activities and mitochondrial abundance. The statistical model used included the fixed effect of RFI group (high and low), gender (heifer and bull) and RFI group × gender interaction. A random sire effect was included in the final model for all traits. Animal day of birth was included in the model as a linear covariate. Model effects were considered statistically significant when type I error rate was less than 5%. Variables (blood metabolites) having multiple observations were analysed using repeated measures ANOVA (PROC MIXED procedure of SAS) with the covariance structure as determined by the Bayesian Information criterion. Terms for RFI group, gender and sampling day and their interactions were included in the model. Differences in RFI group were determined by F-tests using type III sums of squares. The PDIFF option and the Tukey test were applied as appropriate to evaluate pairwise comparisons between RFI group means. The degrees of freedom method used was Kenward-Rogers and an unstructured covariance structure was chosen. Data were considered statistically significant when $P<0.05$ and considered a tendency towards statistical significance when $P<0.10$.

2.6.2 Effect of RFI grouping on gene expression in adipose tissue

In Chapter 4 data were analysed using GeNorm (GenEx 5.2.1.1; MultiD Analyses, Gothenburg, Sweden). GeNorm is model based approach software that measures the overall stability of the tested reference genes by calculating the intra- and intergroup CV and combining both coefficients to give a stability value (M Value). The lower the M value, the higher the stability in gene expression across all samples. An M value of 1.5 is specified as the default minimum coefficient by the GeNorm program. In the present study, *TBP* and *UTX2* were selected as the most suitably stable reference genes. GenEx 5.2.1.3 was used for efficiency correction of the raw cycle threshold value, normalisation to the expression levels of reference genes and calculation of C_t values for each target gene.

Gene expression data were checked for normality and homogeneity of variance using the UNIVARIATE procedure of Statistical Analysis, Software, version 9.3 (SAS Inst., Cary, NC). Data that were not normally distributed were transformed with the TransReg procedure of SAS 9.3. The genes *SCD*, *ACAT1*, *FASN* and *HMG-CoAS* were transformed by rising to the power of lambda which was 2, 1.5, 1.5 and 0.5, respectively. Data were further analysed using the mixed model methodology of SAS 9.3 (PROC MIXED, SAS) with treatment and gender, together with their interaction as the main effects. Animal day of birth was included in the model as a linear covariate.

2.7 Complementary DNA synthesis

2.7.1 cDNA synthesis for qRT-PCR

In Chapter 4 first-strand complementary DNA was synthesised according to manufacturer's instructions using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) which utilised Multiscribe reverse transcriptase. Total RNA (2µg) from each sample was reversed transcribed into cDNA using random hexamers. The converted cDNA was

quantified by absorbance at 260nm, diluted to 50ng/µl working stocks, and stored at -20°C for subsequent analyses.

2.7.2 Non stranded cDNA library preparation and sequencing

cDNA libraries were prepared in Chapter 5 from high quality RNA following the manufacturer's instructions using the Illumina TruSeq RNA sample prep kit (Illumina, San Diego, CA, USA). For each sample, 1 µg of RNA was used for cDNA library preparation. Briefly, mRNA was purified from total RNA and subsequently fragmented. First strand cDNA was synthesised using Superscript II Reverse Transcriptase (Applied Biosystems Ltd., Life Technologies) followed by second strand synthesis using components of the Illumina TruSeq RNA sample prep kit. Adaptors were ligated to the cDNA which was subsequently enriched by 15 cycles of PCR. Libraries were validated on the Agilent Bioanalyser 2100 using the DNA 1000 Nano Lab Chip kit. cDNA concentration was assessed using Nanordrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and samples with >25ng/µl were deemed suitable for further analysis. Following quality control procedures, individual RNA-seq libraries were pooled based on their respective sample-specific 6bp adaptors and sequenced at 100bp/sequence single-end read using an Illumina HiSeq 2500 sequencer. Approximately 22.4 million sequences per sample were generated.

2.7.3 Stranded cDNA library preparation and sequencing

cDNA libraries were prepared in Chapter 6 from high quality RNA following the manufacturer's instructions using the Illumina TruSeq stranded mRNA sample prep kit (Illumina, San Diego, CA, USA). For each sample, 1 µg of RNA was used for cDNA library preparation. Briefly, mRNA was purified from total RNA and subsequently fragmented. First strand cDNA was synthesised using Superscript II Reverse Transcriptase (Applied Biosystems Ltd., Life Technologies) followed by second strand synthesis using components of the Illumina

TruSeq RNA sample prep kit. Adaptors were ligated to the cDNA which was subsequently enriched by 15 cycles of PCR. Libraries were validated on the Agilent Bioanalyser 2100 using the DNA 1000 Nano Lab Chip kit. cDNA concentration was assessed using Nanordrop spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA) and samples with >25ng/µl were deemed suitable for further analysis. Following quality control procedures, individual RNA-seq libraries were pooled based on their respective sample-specific 6bp adaptors and sequenced at 50bp/sequence single-end read using an Illumina HiSeq 2500 sequencer. Approximately 20 million sequences per sample were generated.

2.8 Real-Time quantitative reverse transcription PCR

All primers targeting reference and candidate genes were obtained from a commercial supplier (Sigma-Aldrich Ireland, Dublin, Ireland). Details of primer sets used in Chapter 4 are shown in Table 2.1. Primer 3 (http://frodo.wi.mit.edu/primer3/) and Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) software were used to design primers. Primer specificity was assessed using the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information. Five genes were tested as reference genes across all samples including; Ribosomal protein large PO (*RFLPO*), ubiquitously expressed transcript (*UTX2*), TATA box-binding protein (*TPB*), Topoisomerase II-beta (*TOP2B)* and *β-actin.* Relative real time PCR was carried out using the ABI 7500 Fast real-Time PCR System with Power SYBR® Master Mix (Applied Biosystems, Warrington, UK). Reactions were carried out in a 96 well plate format and prepared in a total volume of 20µl, with 20-100 ng cDNA, 10 µl Power SYBR® master mix and 1 µl of 5-20 µM forward and reverse primer mix. Optimal cDNA concentration, primer (Table 1) efficiencies and concentrations were determined. Non-template controls were included on every plate for each gene product. To minimize variation, all samples included in each analysis were derived from the same cDNA batch, prepared under the same conditions and samples were run in triplicate. Thermal cycling

conditions applied to each assay consisted of an initial Taq activation step at 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s, 60°C for 60 s, followed by an amplicon dissociation stage (95 °C for 15 s, 60 °C for 1min, increasing 0.5 °C/cycle until 95 °C was reached). The specificity of the reaction products was also confirmed by dissociation curve analysis. The efficiency of the qPCR reaction was calculated for each gene by creating a standard curve from 2-fold serial dilutions of cDNA concentration. Amplification efficiencies were determined using the formula E=10^(1/slope), with the slope of the linear curve of cycled threshold (Ct) values plotted against the log dilution of cDNA. Only primers with PCR efficiencies between 80 and 120% were deemed sufficient for subsequent analyses.

Table 2.1. Sequences of bovine oligonucleotide primers used for real-time reverse transcription PCR

Gene symbol	Accession number[1]	Primer sequence (5' -3')[2]
ACACA[4]	NM_174224.2	F: TGTCCGAAACGTCGATTTTTG
		R: ACGACCTGGTTGCTGTGATAGA
HMGCoAR[4]	NM_001105613.1	F: TGAGGGAGAACATTGCTCGT
		R: ACATGATTTCGAGCTGACGC
RFLPO[3]	NM_001012682	F: CAACCCTGAAGTGCTTGACAT
		R: AGGCAGATGGATCAGCCA
UTX2[3]	NM_001037471	F: TGTGGCCCTTGGATATGGTT
		R: GGTTGTCGCTGAGCTCTGTG
TPB[3]	NM_001075742	F: CCTAAAGACCATTGCACTTCG
		R: CTTCACTCTTGGCTCCTGTG
TOP2B[3]	XM_001254709	F: CCGATGATGATGACGACAAT
		R: TGCTATGGGAGATGCTTTGA
B-actin[3]	U39357	F: CTCACGGAGCGTGGCTACA
		R: GCCATCTCCTGCTCGAAGTC

[1] Accession number in the National Centre for Biotechnology Information database (htttp://www.ncbi.nlm.nih/gov/gene).
[2] F=forward primer; R=reverse primer.
[3]Genes used for as reference for qPCR normalisation; *RFLPO*, Ribosomal protein large PO; *UTX2*, Ubiquitously expressed transcript; *TPB*, TATA box-binding protein; Topoisomerase II-beta TOP2B, *B-actin.*
[4]Target genes; *SLC2A4*, solute carrier family 2 member 4; *ACAT1*, Acetyl-CoA Acetyltransferase 1; *HMGCoAS*, Hydroxymethylglutaryl CoA synthase; *SREBP1c*, Sterol regulatory element-binding protein; *FASN*, Fatty acid synthase; *ACLY*, Acetyl citrate lyase; *ELOVL6*, Fatty acid elongase 6; *SCD*, Stearoyl-CoA desaturase-1; *ACACA*, Acetyl-CoA carboxylase; *HMGCoAR*, Hydroxymethylglutaryl CoA reductase.

Table 2.1(contd). Sequences of bovine oligonucleotide primers used for real-time reverse transcription PCR

Gene symbol	Accession number[1]	Primer sequence (5' -3')[2]
GLUT4[4]	NM_174604.1	F: GGGACTGGTACCCATGTACG R: AGGAGGAGTGGCCATAAGGT
ACAT1[4]	NM_001046075.1	F: TCAACGGAGGAGCTGTTTCT R: TGCAAATACTGGCAAGACCA
HMGCoAS[4]	NM_001045883.1	F: AGAACGTCTGCCCTCTTTCA
SREBP1c[4]	NM_001113302.1	F: TACAAGGCTGCTGTGTCCAG F: CTACATCCGCTTCCTTCAGC R: TTCAGCGATTTGCTTTTGTG
FASN[4]	NM_001012669.1	F: TCATCCCCCTGATGAAGAAG R: AACTCCACAGGTGGGAACAG
ACLY[4]	NM_001037457.1	F: CAAGAAGGCAGACCAGAAGG R: CTGGGCGGTACAGCTTAGAG
ELOVL6[4]	NM_001102155.1	F: GGAAAGCAACGAAAGCTGAC R: TGGGTTGTGTGTTTGCTCAT
SCD[4]	NM_173959.4	F: CGACGTGGCTTTTTCTTCTC R: GATACCATGGCACGAGTGTG

[1] Accession number in the National Centre for Biotechnology Information database (htttp://www.ncbi.nlm.nih/gov/gene).
[2] F=forward primer; R=reverse primer.
[3]Genes used for as reference for qPCR normalisation; *RFLPO*, Ribosomal protein large PO; *UTX2*, Ubiquitously expressed transcript; *TPB*, TATA box-binding protein; Topoisomerase II-beta TOP2B, *B-actin.*
[4]Target genes; *SLC2A4*, solute carrier family 2 member 4; *ACAT1*, Acetyl-CoA Acetyltransferase 1; *HMGCoAS*, Hydroxymethylglutaryl CoA synthase; *SREBP1c*, Sterol regulatory element-binding protein; *FASN*, Fatty acid synthase; *ACLY*, Acetyl citrate lyase; *ELOVL6*, Fatty acid elongase 6; *SCD*, Stearoyl-CoA desaturase-1; *ACACA*, Acetyl-CoA carboxylase; *HMGCoAR*, Hydroxymethylglutaryl CoA reductase.

2.9 RNA-sequencing data analysis

2.9.1 Pre-processing, read mapping and gene quantification

2.9.1.1 Chapter 5

FASTQC software (v0.11.5) was used to check the quality of the raw sequencing reads. Input reads were then aligned to the bovine reference genome (UMD3.1) using STAR (v2.5.1). HTSeq (v0.6.1p2) software was used to calculate the number of sequenced fragments overlapping all protein-coding genes from the ENSEMBLv88 annotation of the bovine genome. The number of counts of reads mapping to each annotated gene from HTSeq was then collated into a single file and used for subsequent differential gene expression.

2.9.1.2 Chapter 6

FASTQC software (v0.11.5) was used to check the quality of the raw sequencing reads. Input reads were then aligned to the bovine reference genome (UMD3.1) using Spliced Transcripts Alignment to a Reference (STAR) (v2.5.1). The STAR parameter "quantMode GeneCounts" was used to calculate the number of sequenced fragments overlapping all protein-coding genes from the ENSEMBLv87 annotation of the bovine genome. The number of counts of reads mapping to each annotated gene from STAR was than collated into a single file and used for subsequent differential gene expression.

2.9.2 Expression analysis

2.9.2.1 Chapter 5

The R (v3.4) Bioconductor package, EdgeR (v3.5), which uses a negative binomial distribution model to account for both biological and technical variability, was applied to identify statistically significant differentially expressed genes. The analysis was undertaken using moderated tagwise dispersion. A false discovery rate (FDR) of 0.1 was applied as a threshold

to call genes with differential expression levels. The R (v3.4) Corrplot package was used to visualise the correlation matrices pertaining to the differentially expressed genes (DEGs) passing multiple testing correction for each comparison.

2.9.2.2 Chapter 6

Analysis of gene-level mapped read counts was performed using the Bioconductor package DESeq2. Raw count data was supplied to DEseq2 with no prior normalisation/transformation steps as per DESeq2 guidelines. An internal analysis pipeline (DESeq) was then applied to the raw data, which estimates size factors, dispersion and performs negative binomial GLM fitting and calculates Wald statistics. Separately, read counts were transformed using DESeq2's variance stabilizing transformation (VST) function for visualisation purposes. A false discovery rate (FDR) of <0.1 was applied as a threshold to call genes with differential expression levels. An initial, unsupervised, analysis of the transformed expression data was performed to provide information regarding distribution, trends, and potential outliers. These analyses included Kernel Density Estimation (KDE), Hierarchical Clustering Analysis (HCA), and Principal Components Analysis (PCA). HCA was applied using average linkage and Pearson correlation as distance metric. Using these analyses, samples identified as potential outliers were flagged for further investigation and possible removal from further analysis. Differential expression analysis was performed on each pair-wise contrast as outlined in Table 2.1, with the main emphasis on comparisons 7-8 and 16-18 as per experimental design.

Table 2.2. Pair wise contrasts in which differential expression analyses were performed

Contrast	Base	Test
1	ZG.Hi.CH	H1.Hi.CH
2	H1.Hi.CH	H2.Hi.CH
3	ZG.Hi.CH	H2.Hi.CH
4	H1.Lo.CH	H2.Lo.CH
5	ZG.Lo.CH	H2.Lo.CH
6	ZG.Lo.CH	H1.Lo.CH
7	**H2.Lo.CH**	**H2.Hi.CH**
8	**H1.Lo.CH**	**H1.Hi.CH**
9	**ZG.Lo.CH**	**ZG.Hi.CH**
10	ZG.Hi.HF	H1.Hi.HF
11	H1.Hi.HF	H2.Hi.HF
12	ZG.Hi.HF	H2.Hi.HF
13	H1.Lo.HF	H2.Lo.HF
14	ZG.Lo.HF	H2.Lo.HF
15	ZG.Lo.HF	H1.Lo.HF
16	**H2.Lo.HF**	**H2.Hi.HF**
17	**H1.Lo.HF**	**H1.Hi.HF**
18	**ZG.Lo.HF**	**ZG.Hi.HF**
19	H1.Hi.HF	H1.Hi.CH
20	H1.Lo.HF	H1.Lo.CH
21	H2.Hi.HF	H2.Hi.CH
22	H2.Lo.HF	H2.Lo.CH
23	ZG.Hi.HF	ZG.Hi.CH
24	ZG.Lo.HF	ZG.Lo.CH

H1 = high concentrate, phase 1; ZG = zero grazed grass, phase 2, H2 = high concentrate, phase 2; Hi = High RFI; Lo = Low RFI

For each contrast, one sub-group of libraries with a shared condition (base) acts as a baseline/control group, against which a second sub-group (test) of related libraries is tested. For these tests, the Benjamini and Hochberg adjusted p-value threshold to denote statistical significance in a given gene's expression is set at 0.1 in line with the recommendations of the authors of DESeq2. Results with an adjusted p-value below 0.1 were considered significant.

2.9.3 Pathway analysis

Where applicable in Chapters 5 and 6 biological processes, cellular components and molecular functions that were associated with the differentially expressed genes were identified using the Gene Ontology (GO) enrichment tool of Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.8 [295]. Fisher's exact test was used to determine the enrichment of the GO terms. Annotation clusters and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were assessed using the official gene symbols of the differentially expressed genes (DEGs) and *Bos Taurus* as the species where possible. *P*-values associated with each annotation term inside each cluster are Fisher Exact/EASE Scores. To examine the molecular functions and genetic networks, the RNA-seq data was further interrogated using Ingenuity Pathway analysis (IPA; Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com), a web based software application that enables identification of over-represented biological mechanisms, pathways and functions most relevant to experimental datasets or genes of interest [296, 297]. Data were imported in a flexible format using the gene symbol as the identifier. A core analysis was performed on DEGs. Top networks were identified for all genes from each analysis. A *P*-value for each network was calculated according to the fit of the set of significant genes and the size of the network.

Chapter 3

CHAPTER 3. MITOCHONDRIAL ABUNDANCE AND FUNCTION IN SKELETAL MUSCLE AND LIVER FROM BEEF CATTLE DIVERGENT FOR RFI

3.1 Introduction

Feed provision is the primary cost in beef cattle production systems and consequently there is considerable interest in improved FE as a means of increasing both the economic and environmental sustainability of such enterprises [44]. Residual feed intake, defined as the difference between observed and expected feed intake requirements for maintenance of body weight and growth [32] is considered a useful index to examine the biological mechanisms associated with variation in FE due to its independence from animal performance traits [298]. In addition, RFI is moderately heritable [28, 30, 299] which has led to increased interest in the trait as a candidate for genetic improvement programmes. Research to date has demonstrated that RFI is a complex multifaceted trait [300]. Thus, a solid understanding of its underlying biological control is necessary to further genomic selection processes [301]. While it has been estimated that two-thirds of variation in RFI can be explained by inter-animal variation in energy expenditure [48], there are little data available to substantiate this. Mitochondria account for up to 90% of cellular oxygen consumption and the bulk of ATP synthesis [48] and thus have a major impact on overall FE in mammals. The electron transport chain comprises four multi-protein complexes (CI – CIV; Figure 1.6) which shuttle electrons down the chain creating a proton motive force that drives ATP synthesis [114]. Research indicates differences in activity of the electron transport chain in skeletal muscle [217, 238] and liver [217, 302] of pedigree male broilers and in the skeletal muscle of lambs [230] varying in FE. There is little published information regarding the relationship between RFI and cellular mitochondrial abundance and function in beef cattle [44, 114, 230]. The aim of the current study was to investigate if RFI phenotype has a basis in differential mitochondrial abundance and/or function in two key metabolic organs; liver and muscle, and whether effects were consistent across gender.

3.2 Results

3.2.1 Animal Performance and RFI

Animal performance and feed efficiency data are presented in Table 3.1. There were no interactions amongst the main effects for RFI, DMI, MBW, initial BW, final BW, ADG and BF change ($P>0.05$). Animals of high and low RFI did not differ ($P>0.05$) in initial BW, final BW or ADG. There was an effect of gender ($P<0.05$) on RFI, final BW and ADG. Bulls had greater variation in RFI coefficients, a higher final body weight and a greater ADG than heifers. Heifers and bulls had a mean initial BW (SD) of 372 kg (39.6) and 387 kg (50.6), an ADG of 1.2 kg (0.4) and 1.8 kg (0.3) and DMI of 9.1 kg (0.5) and 9.5 kg (1), respectively, RFI averaged 0.00 for both genderes and ranged from -0.4 to 0.6 kg of DM/d for heifers and -0.5 to 0.5 kg DM/d for bulls. Heifers and bulls ranked as high RFI consumed 10% and 15% more than their low RFI counterparts ($P<0.05$), respectively.

Table 3.1. Summary of phenotypic data of animals divergent for RFI

	RFI Group			Gender			*P*-value		
Trait	High	Low	SD	Bulls	Heifers	SD	RFI	G	RFI × G
No. of animals	17	17	-	18	16	-	-	-	-
DMI, kg/d	9.7	8.8	0.20	9.47	9.1	0.21	0.0025	0.28	0.30
RFI, kg DM/d	0.4	-0.4	0.02	0.03	-0.30	0.01	<0.0001	0.29	0.34
MBW, $kg^{0.75}$	95	95	2.50	97	93	2.51	0.87	0.20	0.79
Initial BW, kg	382	373	16.0	382	372	16.5	0.50	0.62	0.95
Final BW, kg	488	483	16.7	509	462	17.2	0.75	0.011	0.82
ADG, kg	1.5	1.5	0.13	1.8	1.2	0.14	0.68	0.0009	0.62
Back fat change, mm	1.3	1.7	0.24	1.3	1.7	0.25	0.10	0.14	0.62

High RFI is feed inefficient and low RFI is feed efficient.
Metabolic $BW^{0.75}$, kg is determined as $BW^{0.75}$ in the middle of the RFI measurement period which was estimated from the intercept and slope of the regression line after fitting a linear regression line through all metabolic BW (BW0.75) observations.
Back fat change is mean of fat depth at end of intake trial – mean of fat depth at start of intake trial.
G = Gender
SD = Standard Deviation

3.2.2 Blood metabolites

Blood metabolites for the high and low RFI groups are presented in Table 3.2. There were no RFI × gender or RFI × day interactions for any of the metabolites measured. There was a day × gender interaction ($P<0.05$) for urea where heifers had higher concentrations than bulls on both days 1 and 72. Sampling day had an effect ($P<0.05$) on all blood metabolites measured, except for BHB and NEFA. RFI status affected ($P<0.05$) plasma concentrations of NEFA, with concentrations higher in high compared to low RFI animals. Heifers had a higher concentrations ($P<0.05$) of BHB, Albumin and NEFA than bulls at both samplings.

Table 3.2. Blood metabolites of animals divergent for RFI

	RFI			G			D			*P*- value					
Variable	High	Low	SD	Bulls	Heifers	SD	1	72	SD	RFI	G	D	RFI x G	D x G	RFI x D
No.of animals	17.0	17.0	-	18.0	16.0	-	34.0	34.0	-	-	-	-	-	-	-
BHB, mmol/L	0.18	0.22	0.03	0.15	0.25	0.03	0.18	0.21	0.01	0.18	0.02	0.13	0.22	0.06	0.97
Albumin,g/L	34.7	33.7	0.90	32.5	35.9	1.06	31.7	36.7	0.30	0.32	0.01	<0.0001	0.59	0.86	0.56
Creatinine, µmol/L	117	118	5.70	116	120	6.00	110	126	1.72	0.90	0.56	<0.0001	0.45	0.70	0.37
Globulin, g/L	40.6	38.5	2.10	40.4	38.7	1.60	38.6	40.5	1.90	0.30	0.40	0.01	0.80	0.50	0.26
Glucose, mmol/L	4.70	4.80	0.11	4.90	4.60	2.00	4.50	5.00	0.11	0.53	0.09	0.00	0.36	0.17	0.99
NEFA, mmol/L	0.08	0.04	0.02	0.02	0.09	0.02	0.06	0.06	0.01	0.05	0.00	0.18	0.77	0.21	0.35
Total Protein, g/L	75.4	72.2	2.30	73.0	74.6	2.50	70.3	77.3	0.85	0.20	0.51	<0.0001	0.73	0.53	0.24
Triglycerides, mmol/L	0.16	0.17	0.03	0.17	0.15	0.03	0.14	0.19	0.01	0.68	0.67	0.00	0.87	0.48	0.93
Urea, mmol/L	3.90	3.70	0.39	3.40	4.17	0.42	5.10	2.55	0.17	0.71	0.13	<0.0001	0.07	0.01	0.89

RFI = Residual feed intake

G = Gender

D = Day

βHB = Beta-hydroxy butyrate

NEFA= Non esterified fatty acids

SD = Standard Deviation

3.2.3 Citrate Synthase Assay

Results for CS assays in muscle and liver tissue are presented in Table 3.3. There was no RFI × gender interaction and no effect (P>0.05) of RFI or gender on citrate synthase activity in muscle or liver tissue.

3.2.4 Mitochondrial Electron Transport Chain Complex Activities

Results for mitochondrial electron transport chain complex activity assays are presented in Table 3.3. There was an RFI × gender interaction (P<0.05) for CI activity in muscle tissue (Appendix; Figure 9.1), whereby high RFI heifers had higher activity than low RFI heifers (P=0.03) and high RFI bulls tended to have a lower activity than low RFI bulls (P=0.07). There was an effect of RFI status (P<0.05) on CIV activity in the liver tissue, whereby high RFI animals had higher activity than low RFI animals. Gender affected (P<0.01) CI activity in muscle tissue which was manifested as heifers having a higher activity of CI activity than bulls.

3.2.5 Combined activities of electron transport chain complexes

Results for the combined activities of CI and CIII and CII and CIII in muscle and liver tissue are presented in Table 3.4. There was no RFI × gender interaction or effect of gender or RFI status (P>0.05) for activity of CI and CIII or CII and CIII in muscle or liver tissue.

Table 3.3 Activity of Citrate Synthase and Complexes I-IV in muscle and liver of animals divergent for RFI

	RFI Group			Gender			*P*-value		
Enzyme activity	High	Low	SD	Bulls	Heifers	SD	RFI	G	RFI x G
No. of animals	17	17	-	18	16	-	-	-	-
Citrate Synthase (CS) (nmol/min/mg)									
Muscle	138	137	25.9	146	129	26.6	0.98	0.53	0.23
Liver	61	82	19.2	80	63	19.7	0.23	0.39	0.45
Complex activity in muscle (unit/cs)									
CI	0.1	0.1	0.03	0.1	0.2	0.03	0.76	0.0075	0.02
CII	0.4	0.4	0.22	0.2	0.5	0.23	0.74	0.22	0.64
CIII	0.2	0.2	0.04	0.1	0.2	0.05	0.94	0.28	0.47
CIV	0.7	0.4	0.3	0.3	0.8	0.31	0.31	0.2	0.35
Complex activity in liver (unit/cs)									
CI	1.2	0.9	0.3	1.0	1.2	0.31	0.3	0.57	0.24
CII	1.8	1.9	0.55	2.0	1.8	0.56	0.85	0.75	0.41
CIII	0.5	0.4	0.15	0.3	0.5	0.16	0.32	0.2	0.3
CIV	3.2	2.1	0.44	2.4	2.9	0.45	0.02	0.27	0.17

nmol/min/mg = specific activity of enzymes

unit/cs = specific activity normalised to citrate synthase activity

CI-IV = Complex I – IV

RFI = Residual feed intake

Gender = G

SD=Standard Deviation

Table 3.4. Combined activity of Complexes I and III and II and III in muscle and liver of animals divergent for RFI

	RFI Group			Gender			*P*-value		
Enzyme activity	High	Low	SD	Bulls	Heifers	SD	RFI	G	RFI x G
No. of animals	17	17	-	18	16	-	-	-	-
CI+III Muscle (unit/cs)	0.22	0.15	0.09	0.11	0.26	0.09	0.48	0.13	0.68
CII +III Muscle (unit/cs)	0.11	0.09	0.04	0.08	0.11	0.04	0.59	0.52	0.42
CI+III Liver (unit/cs)	0.29	0.21	0.07	0.29	0.21	0.08	0.29	0.34	0.99
CII +III Liver (unit/cs)	2.21	4.26	2.04	2.64	3.82	1.17	0.42	0.66	0.47

cs = citrate synthase

unit/cs = specific activity normalised to citrate synthase activity

CI-IV = Complex I – IV

RFI = Residual feed intake

Gender = G

SD = Standard Deviation

3.3 Discussion

The objectives of this study were, firstly, to examine the effects of RFI status and gender on key performance traits in beef cattle and, secondly, to examine if mitochondrial abundance and functionality in two metabolically important tissue are affected by either gender or RFI status. To the authors knowledge this is the first study to examine the effects of either RFI or gender on mitochondrial abundance and the function of the electron transport chain in muscle or hepatic tissue of beef cattle. The main findings of this study were: firstly, that mitochondrial abundance in either *longissimus dorsi* or liver tissue is not affected by RFI status under conditions used in this study. Secondly, we conclude that altered CI activity in muscle and altered CIV activity in liver may be contributing to variation in energetic efficiency amongst cattle.

The ADG of the heifers (mean 1.2 kg) in this study was lower than the bulls (mean 1.8 kg) which is not surprising as it has been widely documented that heifers have a slower growth rate than bulls when managed similarly [12]. In addition, the majority of studies comparing growth rate across genderes are confounded with 'system related' effects. The current study is unique in that it presents results for a contemporary group of purebred males and females reared similarly from birth through to slaughter. The range in ADG observed was consistent with that of growing beef cattle offered an energy dense diet to appetite [27, 46, 83, 95, 303].

Consistent with our results, many previous studies have found that when managed similarly bulls have a higher growth potential than heifers [304]. While BF accretion was numerically greater for heifers compared with bulls, as might be expected, this difference did not reach statistical significance. Additionally, RFI status, by design did not affect bodyweight change but also did not affect BF accretion for either gender, which is consistent with some [30, 34, 305] but not all studies [55, 93-95]. As per experimental design, no differences in animal performance were observed between the RFI groups, despite a 10 % and 15 % difference in

DMI. This finding is in agreement with other studies examining the relationship between RFI and other productivity traits where RFI was found, phenotypically [300] and genetically [22], to have no effect on weight or growth of beef cattle.

The linear regression model used to compute RFI in the current study accounted for 70% of the variation in DMI. This value is within the range obtained by [27, 44, 83, 95] where energy dense diets were offered. This higher coefficient of determination is not surprising considering animals were offered an energy dense low forage diet. Such diets support high digestibility, low rumen fill and a fast ruminal passage rate compared to more fibrous diets [9].

There is equivocation in the published literature on the relationship between FE status and measures of body fat content [44]. For example, some studies that used the base model (metabolic BW and ADG) to predict DMI have reported either a positive [30] or no relationship [55, 93-95] between FE and carcass fatness traits. In the current study we included an adjustment for BF accretion in our DMI prediction model. The conflicting literature regarding the relationship between RFI and subcutaneous back fat depth may be due to the variation in the extent and timing of fat deposition in different breeds and also differences in the site selected for ultrasonic measurements [7]. As expected BF gain was numerically higher for heifers compared with bulls though this bordered (P=0.10) on statistical significance.

Measuring the systemic concentration of metabolites can contribute to the characterisation of the metabolic state of animals divergent for phenotypic RFI [66, 254, 298]. Metabolites are typically influenced by environmental factors such as diet type and physiological status and perhaps even animal genotype. In general, there was little evidence for a meaningful effect of either gender or RFI status on the systemic concentrations of metabolites measured. While day of sampling affected the concentration of a number of metabolites this was generally consistent with the improved nutritional status of the animals following 70 days of free access to an energy dense diet. In the current study, while NEFA concentrations were low but within the normal

range expected for young growing beef cattle, high RFI animals had twice the concentrations of their low RFI contemporaries. Some authors [1, 3] have observed that βHB was higher for high RFI heifers whereas others [2, 16, 17] found no differences which were in agreement with the current study.

To the author's knowledge, this is the first study to quantify mitochondrial abundance in metabolically important tissues within the context of an RFI model in cattle. Citrate synthase is a vital enzyme in the control of the Krebs cycle and its kinetic properties have been shown to be tightly correlated with the taxonomic status of mitochondria [18]. Measuring the activity of this enzyme is accepted as a reliable proxy for mitochondrial abundance, and can be used to normalise mitochondrial functionality, and the use of this assay has been well documented [19]. A relationship between mitochondrial function and feed efficiency has been well documented [2, 20-24]. Therefore, we hypothesised that given the metabolic importance of liver and muscle, and the observations of [306] who concluded that more efficient pedigree male broilers had a higher mitochondrial RNA content, an increase in mitochondrial number in these organs could be contributing to variation in RFI. Despite this, we observed no differences in mitochondrial abundance in muscle or liver in animals divergent for RFI. This led us to further speculate that while mitochondrial number is unaffected, mitochondria may be functioning at different efficiencies between the two phenotypes.

As aforementioned, there is a plethora of information relating to mitochondrial function and RFI across a number of species [2, 6, 23-26]. There is conflict, however, amongst literature reports in that some authors have observed a higher activity of respiratory chain enzymes in various tissues from more efficient animals [23, 24] while others have observed a decrease in activities of these enzymes in more efficient animals [25]. For the majority of these enzyme complexes we did not see any difference in activity indices for either liver or muscle tissue between RFI classifications, which would indicate, that as a whole there is no difference in the function of the electron transport chain between RFI classifications. CI is the initiation point

for the electron transport chain and is the largest enzyme in the chain. CI has previously been found to be associated with feed efficiency at a transcript level in cattle [27]. We did observe an RFI × gender interaction for CI activity in muscle tissue. This observation could indicate that there is a potential relationship between CI and RFI but it may be a function of gender and warrants further investigation. We observed an effect of RFI on CIV in liver, in which high RFI animals had a higher concentration of this enzyme than low RFI animals. This is in agreement with Sandelin et al. (2005) [232] and may be an indication that the mitochondria in the less efficient animals have to 'work harder' to maintain energy homeostasis.

Mitochondrial respiration is the product of the combined action of the different components of the electron transport chain. Firstly, the actions of the complexes and secondly the electron carriers, co-enzyme Q_{10} and cytochrome c, are required to transport electrons from CI and CII to CIII and subsequently the electronsare transported to CIV by cytochrome c. In the individual enzyme complex assays the electron carriers were included in the assay reaction as electron acceptors or donors [307]. However, defects in mitochondrial respiration due to changes in the interaction of individual complexes or limited amounts of endogenous electron carriers may be missed under these conditions. For example, Coenzyme Q_{10} deficiency is known to impair mitochondrial function, even in the absence of defects in the mitochondrial complexes [308]. With this in mind, it was decided to carry out specific experiments to assess the combined action of two complexes, that is, CI + III activity or CII + III activity [309]. We observed no differences in the combined activities of the electron carrier complexes, co-enzyme Q_{10} and cytochrome c, between animals divergent for RFI indicating that there were no differences in electron flow through the mitochondrial respiratory chain between the phenotypes. These observations combined with those of the complex assays provide further evidence that the activity of the electron transport chain is not affected by phenotypic RFI of cattle in this study.

In conclusion, our findings highlight, for the first time, that mitochondrial abundance is not related to variation in feed efficiency in cattle. It follows that any changes observed in

mitochondrial activity in muscle and liver tissue are as a result of differences in mitochondrial functionality and not due to the presence of more mitochondria. While our work and the work of others highlight a potential shift in energy metabolism between phenotypes we conclude that it is unlikely that differing potential for cellular mitochondrial functionality is contributing majorly to variation in feed efficiency in cattle. A logistical limitation of the present study was that frozen tissue had to be used and thus the mitochondria in the respective tissues were no longer respiring. Future investigation should focus on work with fresh biopsy samples. This would allow for measurement of mitochondrial respiration, proton leak and ROS production which may be contributing to variation for RFI.

Chapter 4

CHAPTER 4. RESIDUAL FEED INTAKE PHENOTYPE AND GENDER AFFECT THE EXPRESSION OF KEY GENES OF THE LIPOGENESIS PATHWAY IN ADIPOSE TISSUE OF BEEF CATTLE

4.1 Introduction

Increased profitability in livestock production systems can be achieved by reducing the costs of inputs, while maintaining or improving the quantity and quality of outputs [1]. Feed provision accounts for approximately 75% of total variable costs in beef production systems [2]. It is widely accepted that small improvements in FE can have a large influence in the profitability of beef systems, by reducing the overall cost of production [3]. Residual feed intake, first described by Koch et al. (1963) [4], can be defined as the difference between an animal's actual feed intake and that predicted based on the animal's BW and average daily gain (ADG) [5]. Efficient cattle, as classified by RFI, are those that consume less feed than expected for a given BW and ADG. RFI is a moderately heritable trait and considerable genetic variation exists to facilitate selection, making it a prime candidate for biomarker identification [6]. In recent years RFI has been favoured as a measure of FE in beef cattle due to its independence from the production traits used to estimate it [7] and thus the concept is thought to represent inherent differences in basic metabolic processes related to energetic efficiency [44, 66]. Herd and Arthur (2009) suggested that approximately one-third of the biological variation in RFI could be attributed to inter-animal differences in digestion, heat increment of feeding, composition of growth and activity, and the remaining two-thirds could be explained by inter-animal variation in energy expenditure [44, 48].

Adipose tissue (AT) is the predominant anatomic site for lipogenesis in ruminants [10] and through its endocrinological activity, modulates a large variety of processes related to feed intake, energy homeostasis, and whole body physiology [11]. While the influence of feed intake on AT accretion, function and distribution has been widely reported, a lack of knowledge

remains on the biological mechanisms mediating the relationship between AT and variation in the efficiency of feed utilization amongst individual cattle [12].

A relationship between lipogenesis and RFI in cattle has previously been highlighted [9]. In addition, in a study examining major gene networks associated with RFI, it was observed that RFI was associated with lipid and steroid metabolism, in particular lipogenesis and cholesterol metabolism [13]. Altered lipid metabolism in the liver [15] and muscle [16] has previously been associated with variation in RFI, where it was observed that key genes involved in lipid metabolism were elevated in less efficient cattle. Similarly, Weber et al. (2016) concluded that there was a deactivation of the regulatory networks controlling fatty acid metabolism in visceral AT of low RFI animals [14]. Despite these findings, the mechanisms by which lipid metabolism influences RFI status in cattle remain to be elucidated, despite its obvious scientific and economic interest. The current study was undertaken to gain greater insight into the effect of RFI status on the expression of key genes involved in the lipogenic pathway (see Figure 4.1) in AT, and how this may be further influenced by gender. We hypothesised that both RFI status and gender would affect the expression of the selected candidate genes.

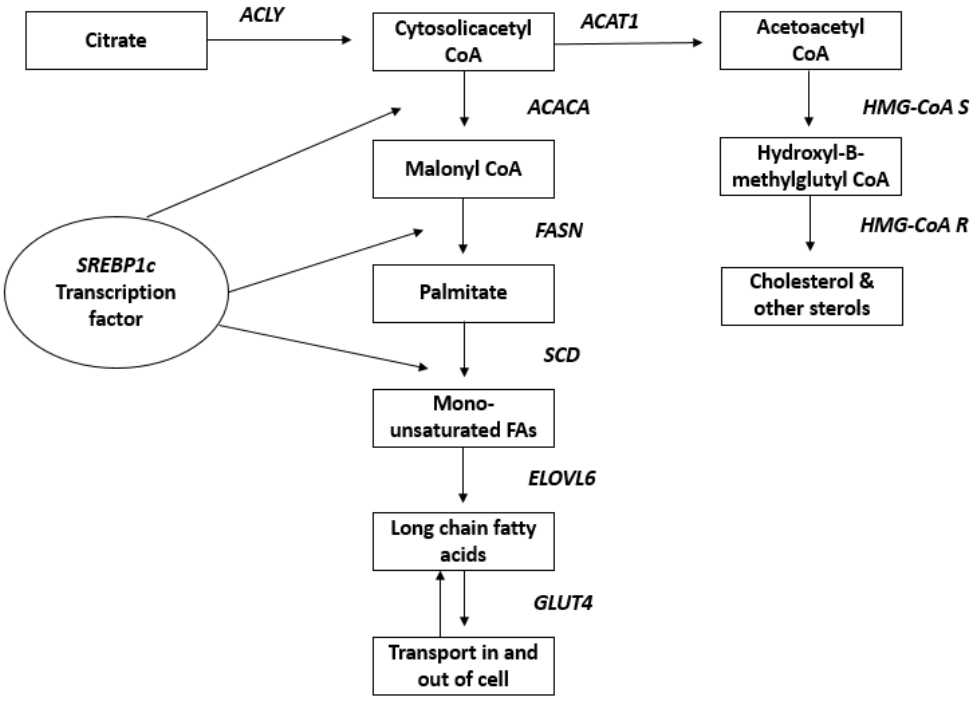

Figure 4.1. Schematic representation of lipogenesis and cholesterol synthesis pathways.

Citrate is cleaved by *ACLY* in the cytosol librerating acetyl CoA. *ACACA* synthesises malonyl CoA from acetyl CoA and malonyl CoA is subsequently converted to palmitate by *FASN*. Following this, *SCD* and *ELOVL6* are responsible for creating long chain fatty acids. Acetyl CoA can also be utilised by *ACAT1* in cholesterol homeostasis pathways which also feature *HMG-CoAS* and *HMG-CoAR*.

4.2 Results

4.2.1 Summary of phenotypic data

The effect of RFI status and gender on DMI, feed efficiency and performance is shown in Table 4.1. Heifers and bulls had a mean initial BW (SD) of 372 kg (39.6) and 387 kg (50.6), respectively. During the feed intake recording period heifers and bulls achieved an ADG (SD) of 1.2 kg (0.4) and 1.8 kg (0.3), and daily DMI (SD) of 9.1 kg (0.5) and 9.5 kg (1), respectively. Residual feed intake averaged 0.00 kg for both groups and ranged from -0.47 to 0.6 kg of DM/d for heifers and -0.50 to 0.59 kg DM/d for bulls. High RFI heifers and bulls consumed 10% and 15% more ($P<0.05$) than their low RFI counterparts, respectively. Animals of high and low RFI did not differ ($P>0.05$) in initial BW, final BW or ADG.

Table 4.1. Summary of phenotypic data of animals divergent for RFI

	RFI Group			Gender			*P*		
Trait	High	Low	SD	Bulls	Heifers	SD	RFI	G	RFI x G
No. of animals	17	17	-	18	16	-	-	-	-
DMI, kg/d	9.7	8.8	0.20	9.4	9.1	0.20	0.0025	0.28	0.309
RFI, kg DM/d	0.4	-0.4	0.02	0.03	-0.3	0.01	<0.0001	0.29	0.34
$MMBW^{0.75}$, kg	95	95	2.5	97	93	2.5	0.87	0.2	0.79
Initial BW, kg	382	373	16.0	382	273	16.5	0.5	0.62	0.95
Final BW ,kg	488	483	16.7	509	462	17.2	0.75	0.011	0.82
ADG, kg	1.5	1.5	0.13	1.8	1.2	0.14	0.68	0.001	0.62
Backfatchange, mm	1.3	1.7	0.24	1.3	1.7	0.25	0.18	0.14	0.62

High RFI is feed inefficient and low RFI is feed efficient.
Metabolic $BW^{0.75}$, kg is determined as $BW^{0.75}$ in the middle of the RFI measurement period which was estimated from the intercept and slope of the regression line after fitting a linear regression line through all metabolic BW (BW0.75) observations.
Back fat change is mean of fat depth at end of intake trial – mean of fat depth at start of intake trial.
P = *P*-value
G = Gender
SD = Standard Deviation

4.2.2 Real-Time quantitative reverse transcription PCR

The effect of divergence in RFI on the expression of genes involved in the lipogenesis pathway in adipose tissue for bulls and heifers is presented in Table 4.2 – 4.4). There was an RFI × gender interaction for the expression of *HMG-CoAS* (P=0.03; Figure 4.2). High RFI bulls tended to have lower expression of *HMG-CoAS* than low RFI bulls (P=0.09), whereas high RFI heifers had higher expression than low RFI heifers (P=0.04) and high RFI heifers (P=0.01). There was an effect of RFI phenotype on the expression of *GLUT4* (P=0.01) in which low RFI animals had a higher expression than high RFI animals. *ACAT1* expression tended to be higher in low RFI bulls than high RFI bulls (P=0.09; Table 4.2). Expression of *ACACA* was higher in low than high RFI heifers (P=0.03; Table 4.4). *ACLY* expression was higher in high compared to low RFI bulls (P=0.02; Table 4.2). There was no effect of RFI on expression of *ELOVL6*, *FASN*, *HMG-CoAR*, *SCD*, and *SREBPC1*. There was an effect of gender on the expression of all genes (P<0.05) excluding *ACLY* (P>0.05) in which heifers had a higher expression of all genes compared to bulls.

Table 4.2. Effect of RFI on the expression of lipogenic genes in adipose tissue from cattle divergent for RFI

	RFI			Gender			*P*-value		
Gene	High	Low	SD	Bulls	Heifers	SD	RFI	G	RFI x G
ACAT1	2.8	3.4	0.39	2.2	4.0	0.42	0.12	0.0002	0.65
ACACA	3.8	4.4	0.40	2.3	5.9	0.42	0.16	<0.001	0.12
ACLY	4.3	3.7	0.70	4.4	3.5	0.70	0.38	0.23	0.32
ELOVL6	3.3	2.9	0.50	2.3	3.8	0.50	0.45	0.01	0.62
FASN	4.2	4.8	0.56	3.8	5.2	0.59	0.38	0.02	0.23
GLUT4	2.3	3.8	0.60	1.4	4.7	0.62	0.01	<0.0001	0.34
HMGCoAR	3.8	4.6	0.94	2.1	6.3	0.99	0.39	0.0002	0.13
HMGCoAS	4.1	5.2	0.88	3.5	5.8	0.94	0.20	0.01	0.03
SCD	5.9	5.9	0.83	4.5	7.3	0.89	0.98	0.003	0.69
SREBPC1	3.9	3.2	0.82	1.9	5.2	0.87	0.40	0.0008	0.54

High RFI is feed inefficient and low RFI is feed efficient.

Gene expression values were calculated by normalizing cycle threshold (Ct) values for target genes to Ct values of the reference genes after adjustment for efficiencies and interplate variation and converted to values relative to the mean Ct value within each data set using GENEX (MultiD Analyses, AB, Gothenberg, Sweden).

G = Gender

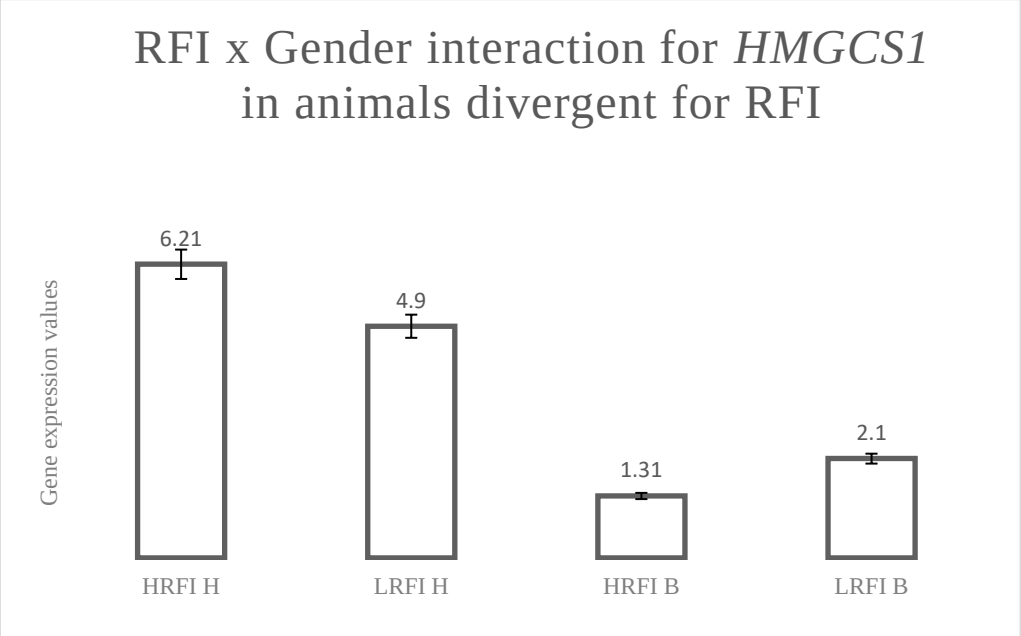

Figure 4.2. RFI x Gender interaction for *HMGCoAS* in animals divergent for RFI. HRFI = High RFI; LRFI = Low RFI; H= Heifer; B = Bull. There was an RFI × gender interaction for the expression of *HMG-CoAS.* High RFI bulls tended to have lower expression of *HMG-CoAS* than low RFI bulls, whereas high RFI heifers had higher expression than low RFI heifers and high RFI bulls.

Table 4.3. Effect of RFI on the expression of lipogenic genes in adipose tissue from bulls divergent for RFI

Gene	HRFI	LRFI	SE	*P*-value
n	9	9		
ACAT1	2.55	3.88	0.68	0.07
ACACA	2.39	2.21	0.48	0.7
ACLY	5.15	4.34	0.29	0.02
ELOVL6	2.74	2.05	0.43	0.13
FASN	4.35	4.23	0.35	0.74
GLUT4	0.96	1.42	0.33	0.19
HMGCoAR	2.46	1.83	0.39	0.13
HMGCoAS	1.31	2.1	0.28	0.02
SCD	4.64	3.65	0.35	0.31
SREBPC1	2.07	2.45	0.41	0.38

HRFI is high RFI (inefficient) and LRFI is low RFI (efficient).
Gene expression values were calculated by normalizing cycle threshold (Ct) values for arget genes to Ct values of the reference genes after adjustment for efficiencies and interplate variation and converted to values relative to the mean Ct value within each data set using GENEX (MultiD Analyses, AB, Gothenberg, Sweden).

Table 4.4. Effect of RFI on the expression of lipogenic genes in adipose tissue from heifers divergent for RFI

Gene	HRFI	LRFI	SE	*P*-value
n	9	9		
ACAT1	3.52	4.38	0.3	0.22
ACACA	4.98	6.63	0.31	0.03
ACLY	3.48	3.24	0.54	0.72
ELOVL6	3.93	3.41	0.41	0.9
FASN	4.21	5.9	0.39	0.17
GLUT4	3.85	5.21	0.52	0.05
HMGCoAR	5.4	6.77	0.81	0.22
HMGCoAS	6.21	4.9	0.58	0.54
SCD	6.81	7.6	0.61	0.89
SREBPC1	4.82	4.55	0.66	0.22

HRFI is high RFI (inefficient) and LRFI is low RFI (efficient).
Gene expression values were calculated by normalizing cycle threshold (Ct) values for target genes to Ct values of the reference genes after adjustment for efficiencies and interplate variation and converted to values relative to the mean Ct value within each data set using GENEX (MultiD Analyses, AB, Gothenberg, Sweden).

4.3 Discussion

Adipose tissue is composed of a variety of cell types, with adipocytes the most predominant. It acts as an energy depot to maintain metabolic homeostasis, ensuring a rapid response to modifications in nutrient availability [22]. It has also been proposed that adipose cell size may be involved in the regulation of feed intake [310]. Furthermore, fat partitioning between fat depots changes greatly with the physiological maturity of cattle with SCAT representing the largest proportion of total body fat [311]. In addition, the energetic cost of laying down SCAT is high, impacting overall energetic efficiency and profitability of cattle. Thus coupled with the fact that SCAT is the most readily measured fat depot, the current study refers to experiments undertaken in SCAT.

Lipogenesis is a key process within adipose tissue in response to increased dietary energy intake [23]. It seems likely that impaired function of this metabolically important organ could contribute to lower energetic efficiency in cattle. The objectives of this study were to examine the premise that differences in the expression of key genes of the lipogenesis pathway in AT contribute to variation in RFI status amongst cattle. While currently there is a dearth of published information on the relationship between the lipogenesis pathway in AT and RFI, our findings add new knowledge on the molecular mechanisms underpinning this trait. To the author's knowledge, this is the first study to examine the combined effects of RFI status and gender on mRNA expression of key genes in adipose tissue of cattle. Genomic selection models do not differentiate on the basis of gender and, if biomarkers are to be of any scientific utility, it is important to determine whether biological processes regulating economically important traits such as RFI are consistent across gender. The main findings of this study were that, firstly, and not unexpectedly, heifers had a higher expression of lipogenesis related genes than bulls where similar genetics, rearing and finishing conditions prevailed. Secondly, we observed differential gene expression of *ACLY* and *HMG-CoAS* between bulls divergent for RFI. Thirdly, we report that heifers divergent for RFI exhibit differential gene expression of *ACACA*. Finally,

we concluded that divergence for RFI across gender affects the gene expression of *GLUT4*, the rate limiting step of glucose uptake from the bloodstream.

It is important to mention that in the current study BF change was included as a variable in the model to predict feed intake, therefore equalising the groups for this trait. Studies have reported that RFI is linked with fat deposition, stating that animals with favourable phenotypes for RFI have leaner carcasses [24], which can lead to unfavourable carcass changes [25, 26]. It is important to correct for BF when selecting for RFI to avoid potential negative effects of co-selecting for leaner animals [27]. In addition, equalising the groups for BF within gender in the current study allowed for examination of the relative metabolic functionality of the AT and its potential variation with respect to RFI over and above subcutaneous-fat deposition.

The results of the current study indicated higher mRNA expression of nine key genes in the lipogenesis pathway in heifers compared to bulls. This result is not surprising as gender differences in adipose deposition and function have been widely documented in the literature, across multiple species [28-30]. In fact, female cattle accumulate more fat than males at the same age [31-33] and it has been observed that heifers have larger adipocytes than bulls of the same BW [30, 32]. We observed a numerically higher deposition of BF in heifers compared to bulls and this observation supports the increased expression of lipogenic genes in heifers [30]. The results of the present study are in agreement with Equinoa et al. (2003) who observed increased activity of lipogenic enzymes in AT in heifers compared to bulls [30]. The comparison between heifer and bull data also provides additional reassurance of our capacity to detect differences in gene expression between the RFI phenotypes should they exist.

Figure 4.1 depicts the role of the genes investigated in the present study in lipogenesis and cholesterol synthesis. We observed that high RFI bulls have greater expression of *ACLY* than their low RFI counterparts. Ruminants rely on gluconeogenesis to meet the majority of their glucose requirements and utilise acetate, produced in the rumen, as their main fuel source for

the initiation of lipogenesis in adipose tissue [34]. Acetate is converted to acetyl-CoA in the cytosol and can be used directly for lipogenesis. Despite this, pyruvate produced through glycolysis is still utilised as an additional source of acetyl-CoA. This acetyl CoA is derived in the mitochondria [35], the inner membrane of which are not permeable to acetyl CoA, causing it to condense with oxaloacetate to form citrate, which can be freely transported into the cytosol. In the cytosol citrate is cleaved by the enzyme *ACLY* liberating acetyl CoA. The activity of *ACLY* is reported to be quite low in ruminants due to the acetate derived cytosolic acetyl-CoA [10, 36]. Nevertheless, this enzyme is an important pre-requisite for fatty acid and cholesterol biosynthesis and occurs when the metabolic state of the animal channels energy from dietary carbohydrates towards lipid storage. The higher expression of this enzyme in high RFI bulls indicates that these cattle are partitioning substrate towards fat deposition as was hypothesised by [24, 37, 38] and Fitzsimmons et al. (2017)[39]. It has also been documented that high starch diets increase *ACLY* expression [40] in cattle and perhaps the higher expression in high RFI bulls in this case is simply a function of a higher DMI of concentrates. It is surprising that we failed to detect a difference in the expression of *ACLY* between high and low RFI heifers. The reason for this is unclear; however, one possible explanation may be differences in insulin sensitivity between genders [41]. *ACLY* is activated by insulin [10] and heifers are more insulin sensitive than bulls [41]. The heifers in the current study were likely at a more advanced stage of physiological maturity than the bulls at the same age and perhaps heifers require less insulin and, in turn, have a lower expression of this enzyme than bulls, accounting for the observed lack of variation between heifers divergent for RFI. This hypothesis could also explain the numerically lower expression of *ACLY* between heifers and bulls (Table 4.2).

Studies have found differences in the expression of the genes *FASN, SCD, ELOVL6* and *SREBP1c* between low and high RFI cattle, in which there was a decrease in gene expression of the regulatory network controlling fatty acid metabolism in adipose tissue of low RFI animals [14, 42, 43]. While this is in agreement with the hypothesis of low RFI animals having less fat

deposition, the present study we did not see a difference in expression levels of these genes between animals divergent for RFI. This discrepancy between studies could be explained by varying levels of divergence for RFI in the animals and different breed types and genders etc. employed between studies or perhaps, more simply, by the fact that the low RFI animals had (numerically) higher fat deposition.

ACACA is a lipid deposition promoting gene that synthesises Malonyl-CoA, a powerful inhibitor of the carnitine/palmitoyl shuttle system for fatty acid β-oxidation and the substrate for *FASN* [44]. The mRNA expression of *ACACA* was higher in low RFI heifers compared to high RFI heifers in the current study, which is not in agreement with the hypothesis of increased lipogenesis in less efficient animals. While these findings are surprising, we did not observe any difference in expression of *ACACA* between bulls divergent for RFI indicating that our observations for heifers may be a result of gender differences and not an effect of RFI status per se.

ACAT1 is a mitochondrial enzyme that catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA. *ACAT1* is a key enzyme in cellular cholesterol homeostasis and a main function of *ACAT1* is believed to guard against the toxic buildup of unesterified cholesterol in the cell membranes [45, 46]. In the present study, we observed expression levels of *ACAT1* to be higher in low RFI bulls, which could indicate that these animals are more efficient at maintaining cholesterol homeostasis which would prevent toxic buildup of unesterified cholesterol. Another potential explanation for the higher levels of *ACAT1* in low RFI bulls could be that these animals have a lower appetite, and thus a lower nutrient intake from which to synthesize cholesterol. It follows that these animals may be compensating for this by producing more cholesterol ubiquitously than their high RFI counterparts.

HMG-CoAS is a mitochondrial enzyme that catalyses the first reaction of ketogenesis, a metabolic pathway that provides lipid-derived energy for various organs during times of

carbohydrate deprivation, such as fasting [47]. In this study the expression of *HMG-CoAS* was higher in low RFI bulls suggesting that these animals could be directing their metabolism towards alternative substrate partitioning such as ketogenesis in order to facilitate their lower feed intake.

The insulin-responsive glucose transporter 4 (*GLUT4*) plays a key role in insulin-stimulated glucose endocytosis and exocytosis in adipose tissue by rapidly translocating from intracellular storage sites to the plasma membrane. Normally *GLUT4* gene expression is increased in in response to states of increased metabolic glucose demand. Impaired regulation of *GLUT4* gene expression and function is associated with insulin resistance and conditions such as type 2 diabetes and obesity in humans and equines [48, 49]. Decreased expression of *GLUT4* in adipose tissue in human obesity and type 2 diabetes has been observed [50, 51]. Furthermore, *GLUT4* expression appears to be upregulated in pigs that are mildly under-nourished [312] but downregulated in lactating goats [313]. Perhaps the most important finding from this study was that there was an effect of RFI across gender on the expression of *GLUT4* in which low RFI animals exhibited a higher expression of this gene compared to their high RFI counterparts. This finding indicates that the decreased expression of *GLUT 4* in the high RFI animals represents less efficient glucose homeostasis and possibly increased insulin resistance in these animals. In addition, Kelly et al. (2011) reported greater levels of circulating plasma insulin in low RFI animals [52] which supports our findings of increased *GLUT 4* in the low RFI phenotype. Furthermore, because changes in *GLUT4* expression can alter many aspects of energy utilization it is likely that *GLUT4* is contributing significantly to variation in RFI.

4.4 Conclusion

Our work together with that of [11, 14, 43] indicate that physiological processes in adipose tissue such as lipogenesis, cholesterol metabolism and overall lipid metabolism are contributing to variation in RFI. Discrepancies between the results reported here and those of other authors

may be a consequence of variation in diet, gender, breed and stage of maturity of the animals employed. This study highlights the relationship between an important metabolic molecule, *GLUT4* and RFI consistent across gender, suggesting it as a potential robust biomarker for RFI. Future work, including global gene expression profiling and targeted proteomics is warranted to investigate this hypothesis further.

Chapter 5

CHAPTER 5. AN EXAMINATION OF THE TRANSCRIPTIONAL CONTROL OF ENERGETIC EFFICIENCY IN SKELETAL MUSCLE AND HEPATIC TISSUE FROM BEEF CATTLE DIVERGENT FOR RESIDUAL FEED INTAKE

5.1 Introduction

Global agriculture is faced with the ambitious challenge of feeding a rapidly increasing population, expected to peak at 9.2 billion by 2075 [1]. As animal feed can account for up to 75% of the variable costs in beef production systems, any reduction of these costs will influence the economic sustainability of the enterprise [2]. Consequently, feed efficiency (dietary nutrient utilisation) in beef cattle is a trait of major economic importance [3]. There is significant phenotypic and genetic variation among beef animals in their ability to convert dietary derived nutrients into saleable product [4-6]. By improving feed efficiency, it is possible to reduce feed intake in cattle while still maintaining overall carcass growth [7].

Residual feed intake, first described by Koch et al. (1963) [8] is a measure of feed efficiency defined as the difference between an animal's actual versus its predicted feed intake, based on average daily gain and metabolic weight [9]. RFI has found favours as a measure of FE due to its independence from the production traits used in its computation and research indicates that variation in RFI reflects variation in basal metabolic processes. Animals with a low RFI (efficient) consume less than their high RFI (inefficient) counterparts for the same level of production [3, 10-16]. Studies examining RFI in growing beef cattle have shown that individual animals, across multiple breed types, can consume up to 20% less feed than their counterparts for the same level of performance [3, 11, 12, 14-20]. Estimates of genetic variation in RFI indicate that the trait is moderately heritable and not antagonistically correlated with other economically important traits [21], making it an ideal candidate for inclusion in a selection index. The challenge being faced, is the reliable and cost-effective identification of feed

efficient animals and subsequent proliferation of their genetics within targeted cattle breeding programmes [7].

The main impediment to genetic progress and adoption of selection strategies based on RFI is the logistics and expense of routine measurement of individual animal feed intake and to a lesser extent BW. It follows that developing accurate predictive biological markers specifically for improved feed efficiency is an attractive alternative to direct measurement on large numbers of animals [6]. Within this context, a better understanding of the biological mechanisms underlying RFI will be fundamental to breeding efforts based on genomic selection.

Muscle and liver are important metabolic tissues with skeletal muscle accounting for over 50% of body weight [13] and liver accounting for 18-25% of total oxygen body consumption [22]. The advent of global transcriptomic analysis provides a powerful tool for a comprehensive insight into the functionality of metabolically important tissues and constituent cellular biochemical processes. Such a holistic approach is of particular relevance to complex, multifactorial trait such as RFI [23-27]. It is estimated that two thirds of the variation in RFI is due to variation in resting energy expenditure [5]. Genes involved in ion transport and binding, membrane binding, transport and catalysis of molecules through the cell membrane, essential mechanisms of nutrient absorption and mitochondrial function have been identified as contributing to variation in RFI in certain breeds of both beef and dairy cattle [23-25, 28]. Despite this, the question over what molecular processes may be controlling RFI in cattle and to what degree these are consistent across breed, gender, physiological stage of maturity and/or production system remains. In particular, given the significant physiological difference in body growth and development between male and female cattle post puberty, it is important to understand the key genes and biological processes regulating FE between the genders during this economically important period, if ubiquitously relevant biomarkers for improved feed efficiency are to be identified.

The primary objective of the present study was to identify the regulatory genes and molecular pathways underlying the expression of the RFI trait in purebred heifers and bulls of the same breed and managed similarly from birth, by characterising the hepatic tissue and skeletal muscle transcriptome, using RNA-seq technology.

5.2 Results

5.2.1 Animal performance

The performance of the entire population of cattle have been described in Chapter 3 (Table 3.1) while details of live weight gain, feed intake, and animal performance of the subpopulation of cattle used in this study are presented in Tables 5.1 and 5.2 for bulls and heifers, respectively. Briefly, bulls and heifers had a mean ADG of 1.8 kg and 1.3 kg and DMI of 9.3 kg and 9.2 kg respectively during the RFI measurement period. Residual feed intake averaged -0.04 and 0.04 and ranged from -1.92 to 1.04 kg of DM/d and -0.43 to 0.68 of DM/d for bulls and heifers respectively. Bulls and heifers ranked as high RFI consumed 10 and 15 % more than their low RFI counterparts ($P<0.05$), respectively. Bulls of high and low RFI did not differ ($P>0.05$) in initial BW, final BW or ADG and likewise for heifers.

Table 5.1. Summary of phenotypic data of bulls at end of feed intake trial

Trait	High	Low	SD	*P*-value
No. of animals	5	5	-	-
DMI,kg DM/d	10.1	8.4	0.46	0.02
RFI, kg DM/d	0.6	-0.7	0.06	0.009
Metabolic $BW^{0.75}$,kg	96.3	94.5	5.2	0.74
Initial BW,kg	375.1	376.9	34.6	0.9
Final BW,kg	511.8	496.6	29	0.62
ADG, kg/d	1.9	1.7	0.3	0.46
Backfatchange[3], mm	1.5	1.5	0.33	0.63

High RFI is inefficient and low RFI is efficient.
Metabolic $BW^{0.75}$, kg is determined as $BW^{0.75}$ in the middle of the RFI measurement period which was estimated from the intercept and slope of the regression line after fitting a linear regression line through all metabolic BW (BW0.75) observations.
Back fat change is mean of fat depth at end of intake trial – mean of fat depth at start of intake trial.
SD = Standard Deviation

Table 5.2. Summary of phenotypic data of heifers at end of feed intake trial

Trait	High	Low	SD	*P*-value
No. of animals	5	5	-	-
DMI,kg DM/d	9.8	8.6	0.26	0.03
RFI, kg DM/d	0.4	-0.3	0.04	<0.0001
Metabolic $BW^{0.75}$,kg	91	92	3.5	0.89
Initial BW,kg	368	361	20.7	0.73
Final BW,kg	435	347	26.5	0.93
ADG, kg/d	1.3	1.4	0.2	0.59
Back fat change, mm	1.3	1.2	0.4	0.16

High RFI is inefficient and low RFI is efficient.
Metabolic $BW^{0.75}$, kg is determined as $BW^{0.75}$ in the middle of the RFI measurement period which was estimated from the intercept and slope of the regression line after fitting a by linear regression line through all metabolic BW (BW0.75) observations.
Back fat change is mean of fat depth at end of intake trial – mean of fat depth at start of intake trial.
SD = Standard Deviation

5.2.2 RNA-seq read alignment and differential gene expression

RNA sequencing data was successfully created for all samples with approximately 22.4 million sequences per sample generated across all samples (skeletal muscle and hepatic tissue). On average approximately 85% of reads aligned to the bovine reference genome (UMD3.1) across both tissues analysed. Figures 5.1-5.4 display multi – dimensional scaling plots based on normalised expression values in skeletal muscle and hepatic tissue from both high and low RFI heifers and bulls. Figures 5.5-5.8 display MA plots representing fold change versus expression strength of all DEG analyses. Despite an average of 6,000 genes being expressed in skeletal muscle tissue from bulls (Figures 5.3 and 5.7) no DEG were detected between divergent RFI phenotypes. A total of 5 genes (P<0.001; FDR <0.1; Figures 5.1 and 5.5) were identified to be differentially expressed in skeletal muscle tissue between high and low RFI heifers with all 5 transcripts being upregulated in the low RFI phenotype. Figure 5.9 provides a visual representation of the correlation matrix pertaining to the DEG from heifer skeletal muscle tissue, indicating all DEG to be highly correlated with each other. Among the (approximately) 7,000 genes detected in hepatic tissue from heifers (Figures 5.2 and 5.6), none were found to be differentially expressed. A total of 11 genes (P<0.001; FDR <0.1; Figures 5.4 and 5.8) were identified to be differentially expressed in hepatic tissue between high and low RFI bulls with 8 transcripts upregulated and 3 downregulated in the low RFI phenotype. Figure 5.10 provides a visual representation of the correlation matrix pertaining to the DEG from bull hepatic tissue, highlighting the most correlated genes. Further details of the DEG are provided in Tables 5.3 and 5.4.

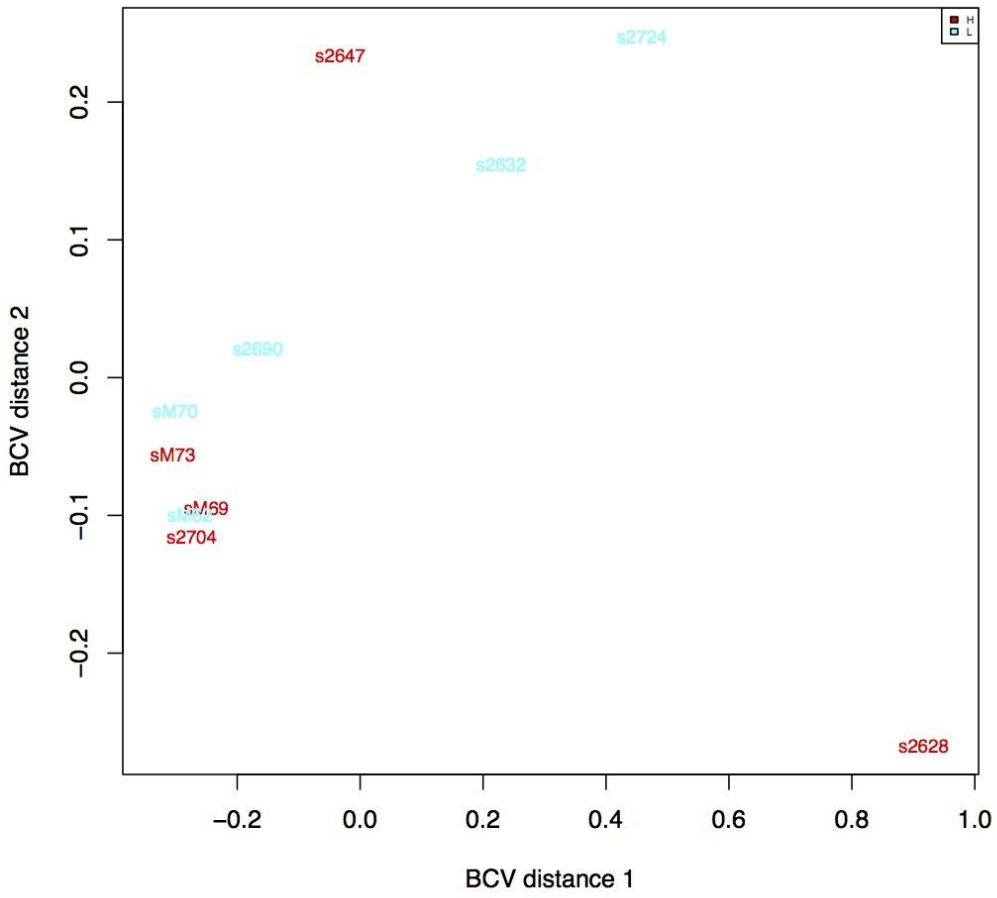

Figure 5.1. Mds plot based on normalised expression values in muscle from high and low RFI heifers. Plot in which distance corresponds to the biological coefficient of variation, with some separation of high (red) and low (blue) RFI groups in gene transcript abundance reads in heifer muscle

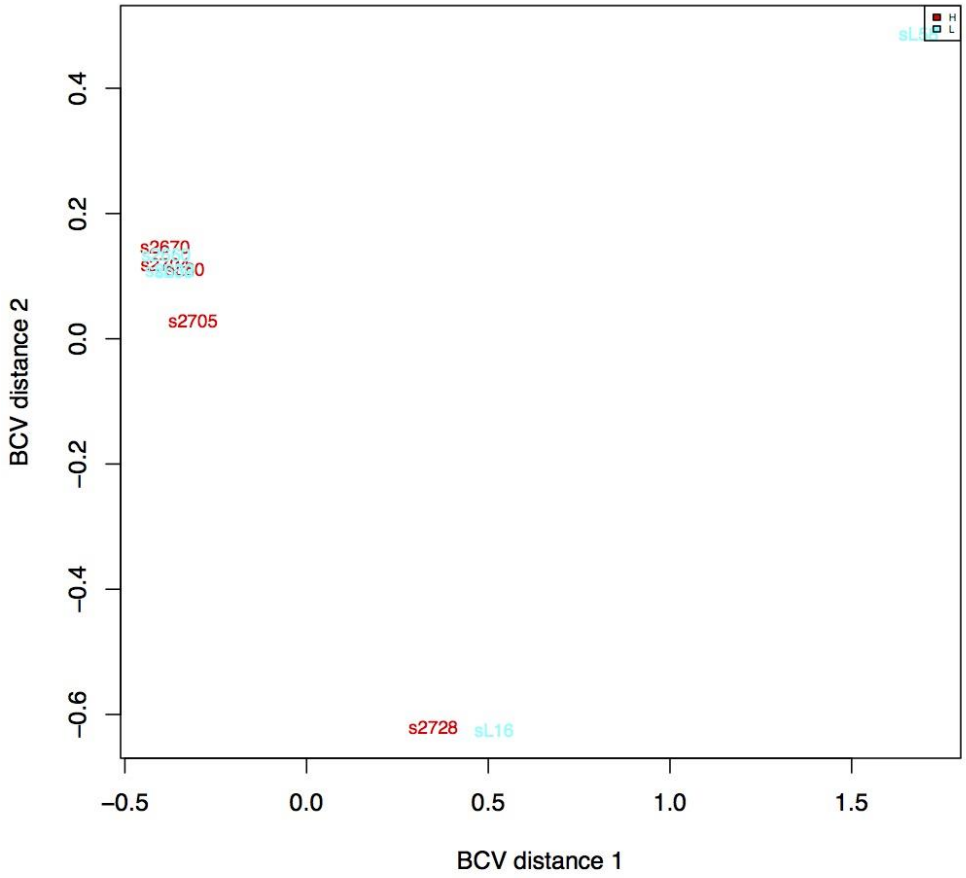

Figure 5.2. Mds plot based on normalised expression values in liver from high and low RFI heifers. Plot in which distance corresponds to the biological coefficient of variation, with no clear separation of high (red) and low (blue) RFI groups in gene transcript abundance reads in heifer liver

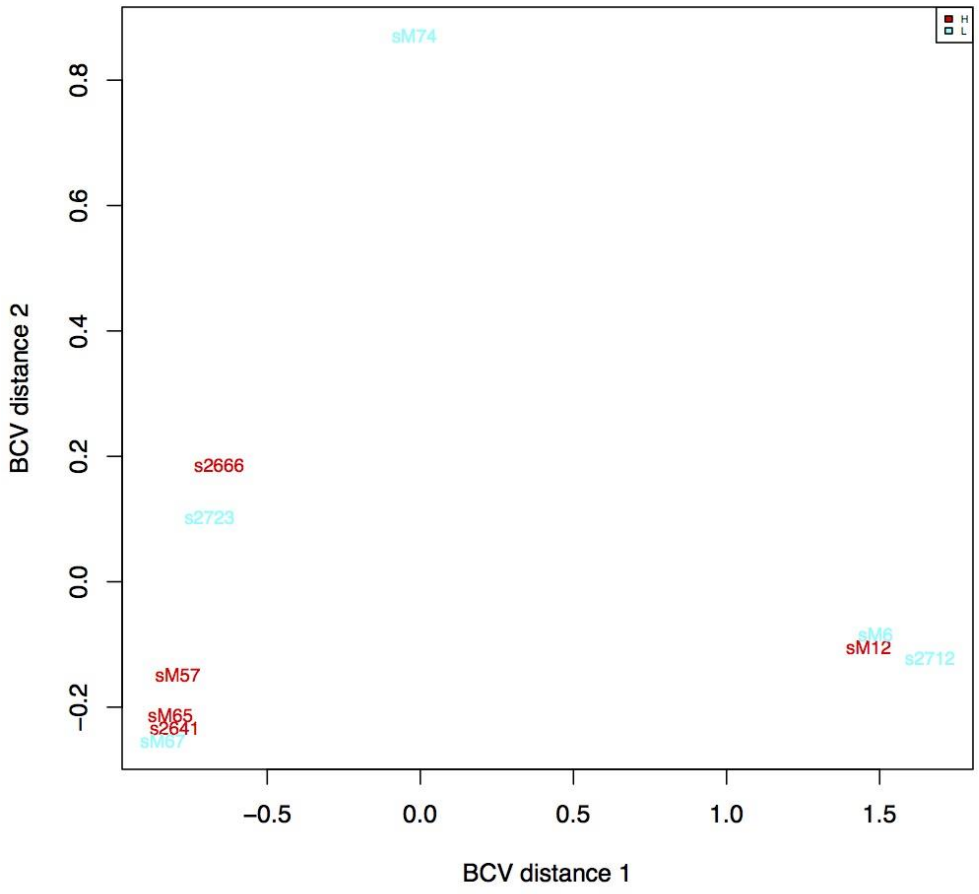

Figure 5.3. Mds plot based on normalised expression values in muscle from high and low RFI bulls. Plot in which distance corresponds to the biological coefficient of variation, with no separation of high (red) and low (blue) RFI groups in gene transcript abundance reads in bull muscle

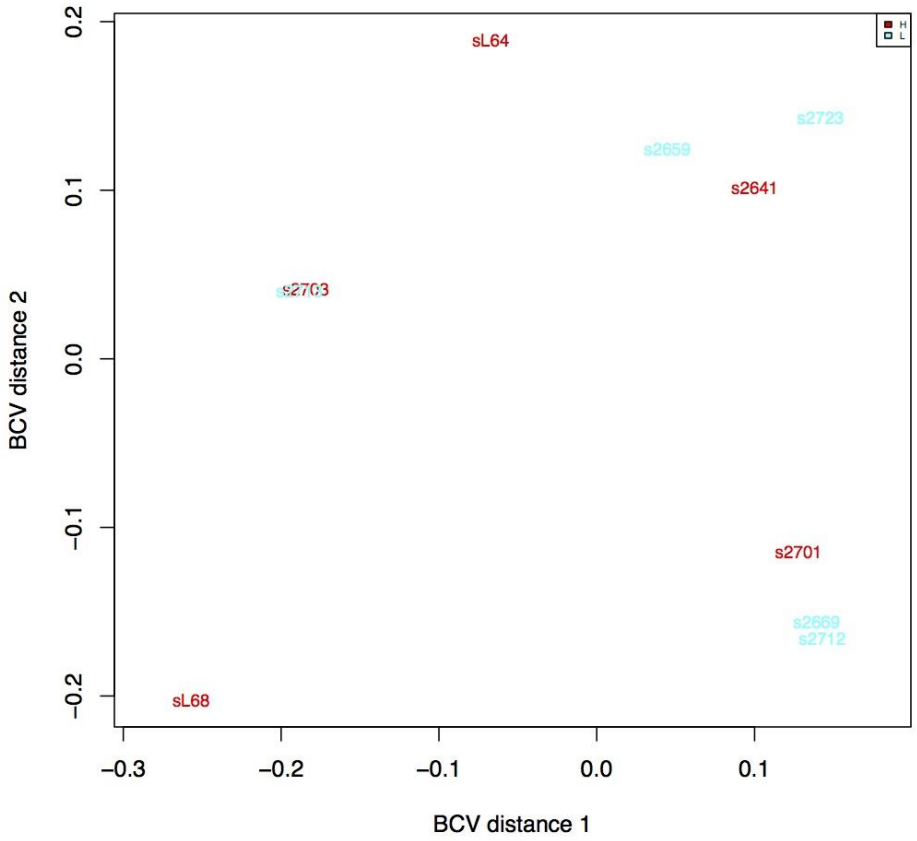

Figure 5.4. Mds plot based on normalised expression values in liver from high and low RFI bulls. Plot in which distance corresponds to the biological coefficient of variation, with some separation of high (red) and low (blue) RFI groups in gene transcript abundance reads in bull liver

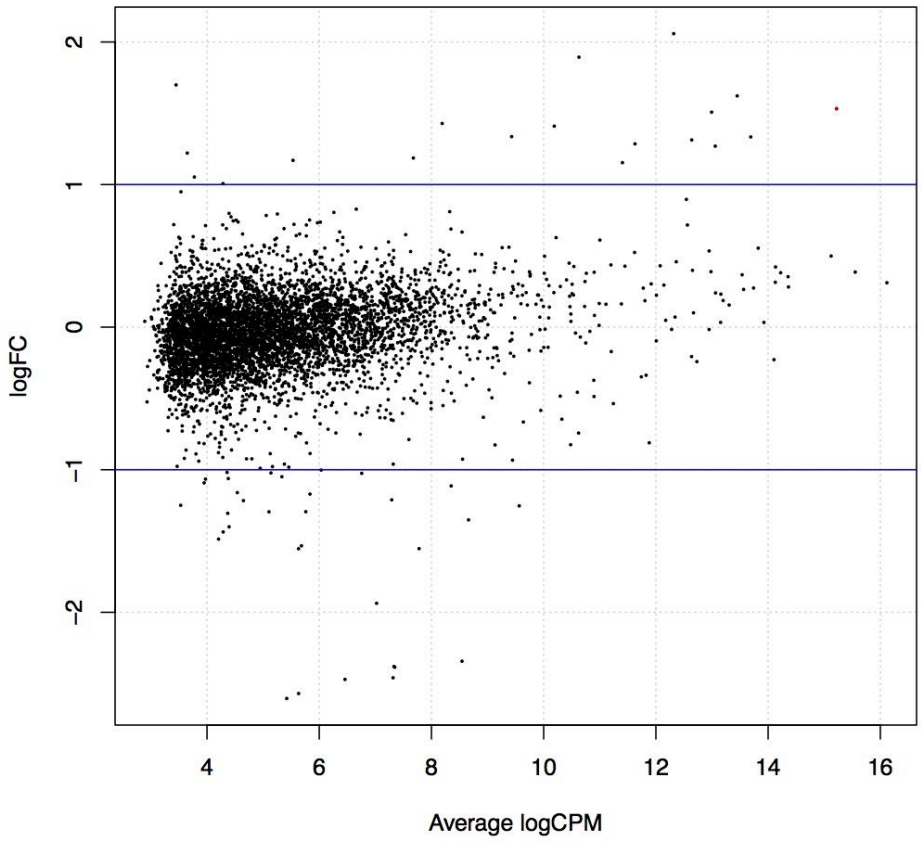

Figure 5.5. MA plot of gene expression changes in muscle tissue from high and low RFI heifers. MA plot reporting fold changes against average expression. The MA plot indicates log2 fold changes for genome-wide genes (Y-axis) plotted against their respective log2 average expression (X-axis). The red dots represent significantly differentially expressed genes in muscle between the low and high RFI heifers

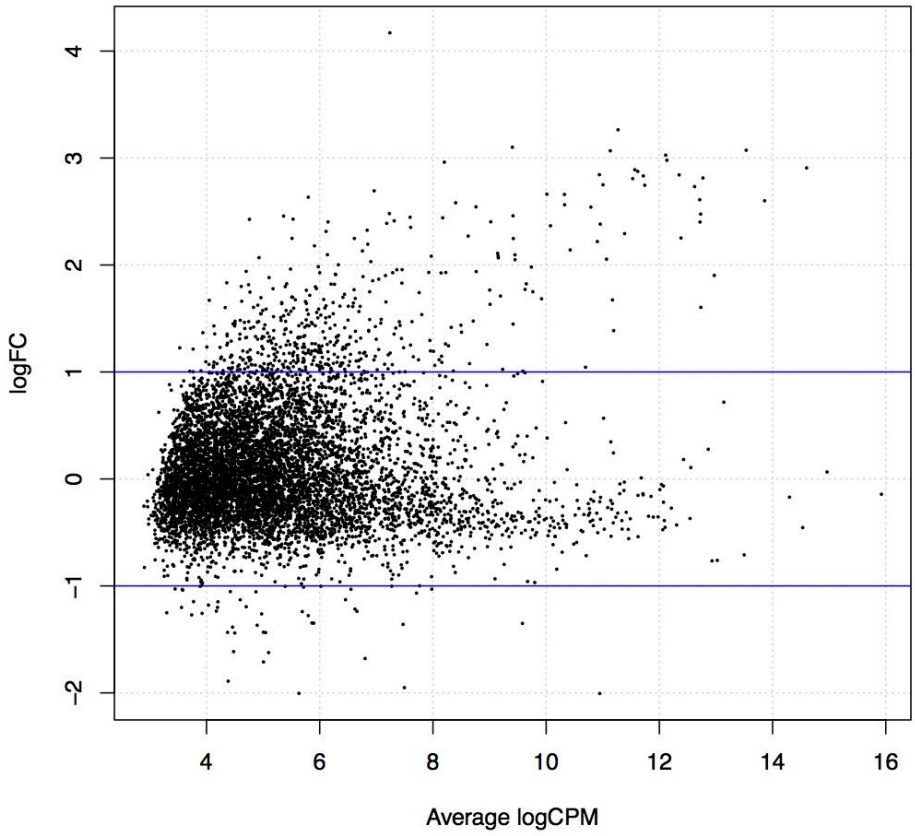

Figure 5.6. MA plot of gene expression changes in liver tissue from high and low RFI heifers. MA plot reporting fold changes against average expression. The MA plot indicates log2 fold changes for genome-wide genes (Y-axis) plotted against their respective log2 average expression (X-axis). The red dots represent significantly differentially expressed genes in liver between the low and high RFI heifers

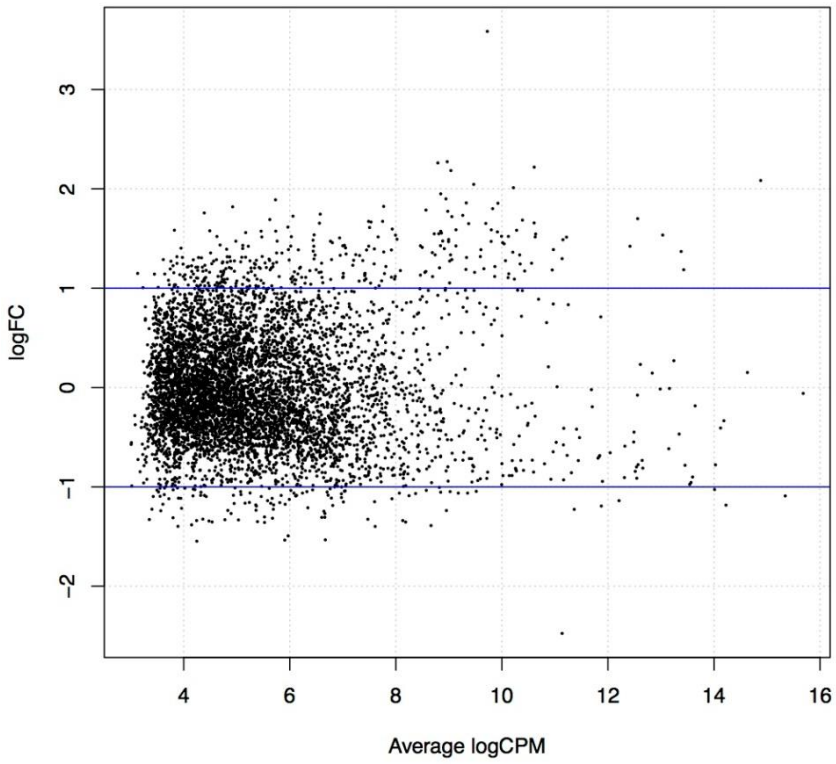

Figure 5.7. MA plot of gene expression changes in muscle tissue from high and low RFI bulls. MA plot reporting fold changes against average expression. The MA plot indicates log2 fold changes for genome-wide genes (Y-axis) plotted against their respective log2 average expression (X-axis). The red dots represent significantly differentially expressed genes in muscle between the low and high RFI bulls

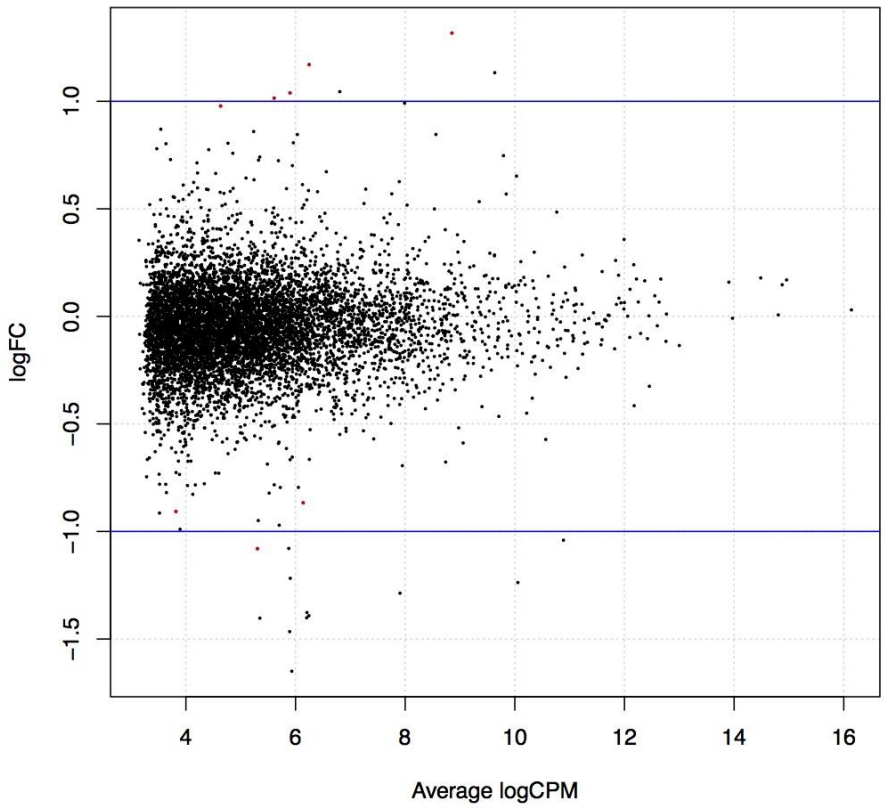

Figure 5.8. MA plot of gene expression changes in liver tissue from high and low RFI bulls.

MA plot reporting fold changes against average expression. The MA plot indicates log2 fold changes for genome-wide genes (Y-axis) plotted against their respective log2 average expression (X-axis). The red dots represent significantly differentially expressed genes in liver between the low and high RFI bulls

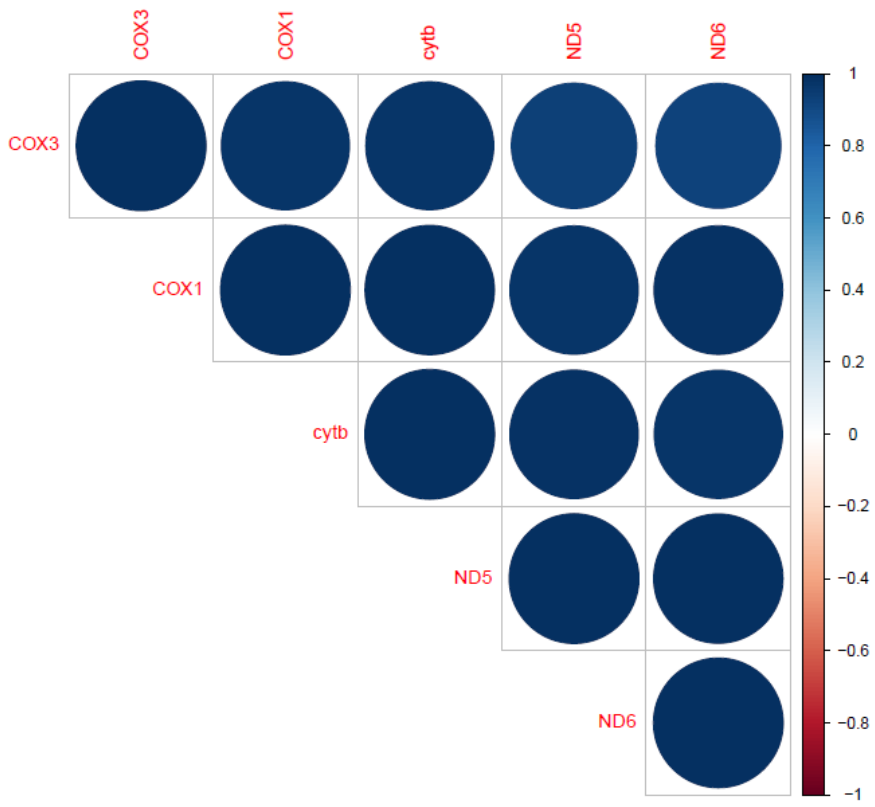

Figure 5.9. Correlogram highlighting the correlation between DEG in muscle tissue from high and low RFI heifers. Visual representation of the correlation matrix highlighting the most correlated DEG in muscle tissue from high and low RFI heifers. Blue circles (correlation value 1) indicate a high correlation between DEG.

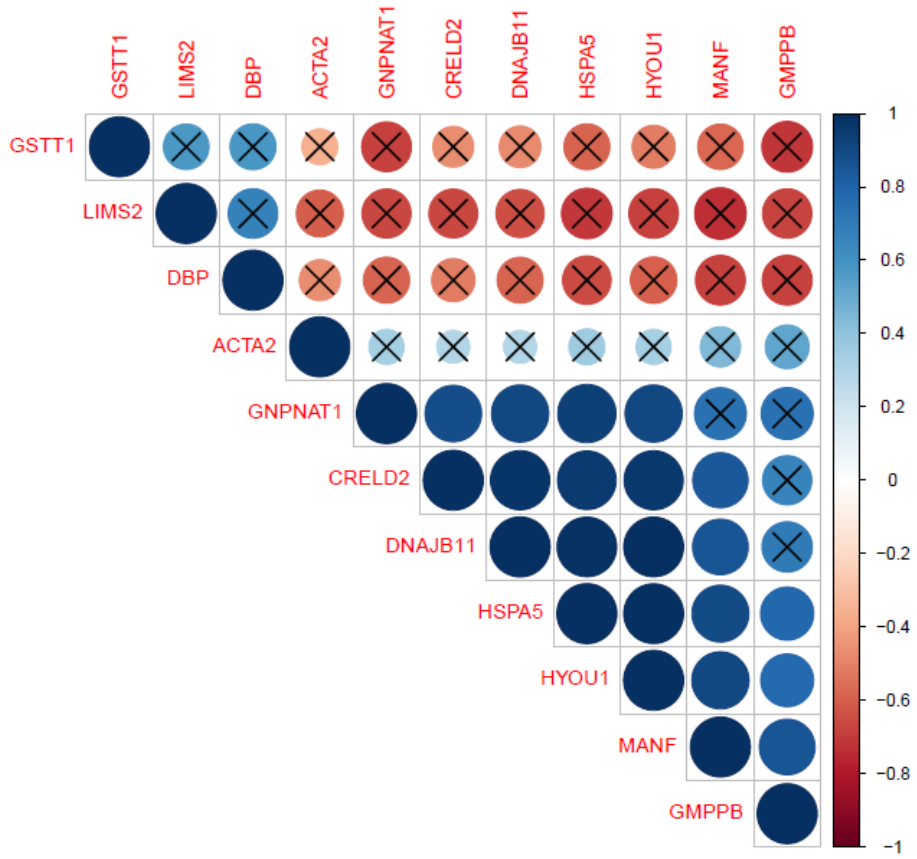

Figure 5.10. Correlogram highlighting the correlation between DEG in liver tissue from high and low RFI bulls. Visual representation of the correlation matrix highlighting the most correlated DEGs in liver tissue from high and low RFI bulls. Blue circles (correlation value 1) indicate a high correlation between DEG while red circles (correlation value -1) indicate no correlation between DEG.

Table 5.3. Differentially expressed genes in the skeletal muscle of heifers divergent for RFI

Ensemble Gene ID	Symbol	Log^2FC	FDR	*P*-value
ENSBTAG00000043561	*COX1*	1.532	0.042	7.62E-06
ENSBTAG00000043563	*ND5*	2.0581	0.066	2.37E-05
ENSBTAG00000043546	*ND6*	1.8935	0.072	3.87E-05
ENSBTAG00000043550	*cytb*	1.6219	0.081	5.84E-05
ENSBTAG00000043560	*COX3*	1.3339	0.082	7.43E-05

Table 5.4. Differentially expressed genes in the hepatic tissue of bulls divergent for RFI

Ensemble Gene ID	Symbol	Log^2FC	FDR	*P*-value
ENSBTAG00000007662	*HSPA5*	1.3668	0.002	3.19E-07
ENSBTAG00000006262	*LIMS2*	-0.8168	0.007	2.15E-06
ENSBTAG00000047801	*CRELD2*	1.038	0.007	2.88E-06
ENSBTAG00000010322	*HYOU1*	1.2267	0.01	5.35E-06
ENSBTAG00000031797	*MANF*	1.0976	0.021	1.43E-05
ENSBTAG00000000170	*GSTT1*	-0.8441	0.025	2.05E-05
ENSBTAG00000014614	*ACTA2*	1.075	0.026	2.48E-05
ENSBTAG00000003151	*DNAJB11*	0.9105	0.026	2.86E-05
ENSBTAG00000006754	*DBP*	-1.0226	0.045	5.49E-05
ENSBTAG00000032026	*GMPPB*	0.6323	0.055	7.43E-05
ENSBTAG00000005344	*GNPNAT1*	0.6199	0.08	0.00012

5.2.3 Differentially expressed genes and pathway analysis

Functional analysis using both the up- and down-regulated genes was performed using DAVID software and revealed that in hepatic tissue from bulls the DEG were related to metabolism of amino sugars and nucleotides, post-translational modification, protein turnover and chaperones and protein processing in the endoplasmic reticulum (Table 5.6). Functional analysis using DAVID in heifer skeletal muscle indicated the DEG to be involved in oxidative phosphorylation and mitochondrial homeostasis (Table 5.5). All DEG were successfully mapped to a molecular or biological pathway and/or category in the IPA database. DEG were analysed and separated according to their biological function within IPA. The top canonical pathways affected by RFI in heifer skeletal muscle tissue are presented in Table 5.7 and these include mitochondrial function and oxidative phosphorylation. The top canonical pathways affected by RFI in bull hepatic tissue are presented in Table 5.8. These include aldosterone signalling, GDP-mannose biosynthesis, nuclear factor erythroid 2-related factor 2 (NRF2)–mediated oxidative stress response, integrin linked kinase (ILK) signalling and eukaryotic initiation factor 2 (EIF2) signalling.

Table 5.5. Enriched GO functions in skeletal muscle from heifers divergent for RFI as identified by DAVID

Function	*P*-value	BH
Cluster 1 (Enrichment Score: 4.28)		
Respiration	3.00E-09	3.50E-08
Oxidative phosphorylation	1.10E-07	7.80E-07
Respiratory chain	1.50E-07	2.30E-06
Parkinson's disease	1.60E-07	5.40E-07
Electron transport	4.80E-07	3.80E-06
Mitochondrion	1.80E-06	9.60E-06
Ubiquinone	7.90E-06	3.20E-05
Mitochondrion inner membrane	6.90E-04	2.20E-03
mitochondrion	7.20E-04	4.30E-03
Metabolic pathways	7.30E-04	1.30E-03
Transport	8.50E-04	2.30E-03
mitochondrial inner membrane	2.10E-03	8.30E-03
Oxidoreductase	2.90E-03	6.60E-03
integral component of membrane	5.40E-03	1.60E-02
Transmembrane helix	5.90E-03	1.20E-02
Transmembrane	6.00E-03	1.10E-02
Membrane	1.00E-02	1.60E-02
Cluster 2 (Enrichment score:3.03)		
hydrogen ion transmembrane transport	3.30E-05	3.00E-04
cardiac skeletal muscle contraction	6.50E-04	1.50E-03
Non- alcoholic fatty hepatic tissue disease	2.60E-03	3.60E-03
Alzheimer's disease	3.20E-03	3.70E-03
Huntington's disease	4.00E-03	4.00E-03

BH = Benjamini-Hochberg

Table 5.6. Enriched GO functions in hepatic tissue from bulls divergent for RFI as identified by DAVID

Function	*P*-value	BH
Cluster 1 (Enrichment Score: 0.7)		
Signal	1.60E-01	9.50E-01
Disulfide bond	2.00E-01	9.20E-01
Signal peptide	2.50E-01	1.00E+00

BH = Benjamini-Hochberg

Table 5.7. Canonical pathways associated with divergence for RFI in heifer skeletal muscle

Canonical Pathway	*P*-value
Mitochondrial function	3.18E-11
Oxidative Phosphorylation	3.26E-09

Table 5.8. Canonical pathways associated with divergence for RFI in bull hepatic tissue

Canonical Pathway	*P*-value
Aldosterone signalling in epithelial cells	2.11E-03
GDP-mannose biosynthesis	2.54E-03
NRF2-mediated oxidative stress	2.83E-03
ILK signalling	2.92E-03
EIF2 signalling	3.70E-03

5.3 Discussion

The main observation of the current study is the inconsistent effect of RFI across (i) gender and (ii) tissue for DEG profiles despite a 10% and 15% difference in DMI between high and low RFI heifers and bulls, respectively. In the current study, while both genders were of similar age and were reared under the same conditions, it is apparent that both genders were at distinct physiological stages at the time of sample collection. Our results indicate that it will be unlikely that the same genetic variants will be accurate predictors of genetic potential for RFI across gender. Despite this, we determined possible molecular mechanisms and biological functions influencing RFI in beef cattle within tissue and gender subgroups. These data highlight a relationship between RFI and the transcriptomic networks involved in mitochondrial function in the skeletal muscle of heifers and evidence for a relationship between RFI and the aldosterone signalling pathway and the NRF2 mediated oxidative stress pathway in hepatic tissue of bulls.

Variation in total energy expenditure of animals of the same breed, similar genetics and management conditions may arise from differences in physiological processes such as ion pumping, mitochondrial proton leak, protein uncoupling, hormonal control of metabolism and lipid metabolism [314]. Gene expression is a key source of variation between individuals and may be used to identify candidate genes and pathways associated with energy expenditure in cattle.

The metabolic activities of the liver and skeletal muscle are essential for the overall homeostasis and efficiency of an animal with both organs being highly abundant in mitochondria. Muscle accounts for approximately 50% of body mass and 25% of basal metabolic activity of an animal and plays an important role in resting energy expenditure [315]. The liver is a highly oxidative organ accounting for 18-25% of total oxygen body consumption that is responsible for metabolising lipids, proteins and carbohydrates into biologically useful molecules [316]. We hypothesised that due to the metabolic importance of these organs, that variation in FE and

energy expenditure (measured here using the concept of RFI) is likely to be reflected in the transcriptome of tissue from these organs. Understanding the essential biological processes contributing to variation in RFI is critical to elucidating the genetic basis, if any, for this trait.

Our analysis identified many DEG between both heifers and bulls divergent for RFI in skeletal muscle and hepatic tissue. However, while DEG were detected in bull skeletal muscle and heifer hepatic tissue, these comparisons failed to pass the relatively conservative statistical rigour employed in our analysis and obtain an adequate FDR value. The animals used in the current study were from a purebred, well-characterised herd, reared as a contemporary group from birth with similar genetics, thus permitting a more equitable comparison of the effect of phenotypic RFI ranking. The inconsistency in DEG profiles across gender can be attributed to the differences in the physiological stage of development between the two genders during the feed intake period. The current study failed to establish any uniformity between the two genders, thus the likelihood of identifying robust consistent biomarkers for crossbred cattle of varying stages of maturity and rearing status is highly questionable. These results are common for RFI studies due to a lack of a definite treatment effect i.e. RFI, calculated within a given population, reflects natural variation within that population. Indeed, many studies of feed efficiency in cattle have published DEG lists that did not pass multiple testing correction, as reflected by an FDR value greater than 0.1 [317-319]. With RNA-seq, quite often what makes these experiments attractive is their high throughput nature, however this is what causes opportunities for false positives where many candidates must be statistically tested in multiple comparisons [320]. The decision lies in the trade-off between the scientific costs associated with a false positive results versus the benefit of identifying a novel gene in the biological model being examined. With this in mind, for the purpose of this study, we have decided to only discuss results that obtained an appropriate FDR value; i.e. heifer skeletal muscle and bull hepatic tissue gene expression profiles.

We identified 5 genes that were statistically significantly differentially expressed in skeletal muscle of high and low RFI heifers. The low number of differentially expressed genes identified is consistent with the lack of clear separation in gene expression profiles between low and high RFI heifers observed on the Multidimensional scaling plot (Figure 5.1) and consistent with the findings, of other previously published RFI based studies [289, 301, 321]. However, among the 5 differentially expressed genes, all 5, *COX1 (cytochrome c oxidase subunit 1), ND5 (NADH-dehydrogenase 5), ND6 (NADH-dehydrogenase 6), CYTB (cytochrome b),* and *COX3 (cytochrome c oxidase subunit 3),* were upregulated in low RFI heifers compared to their high RFI counterparts. Interestingly all of these genes are components of the electron transport chain (ETC) in the mitochondrial inner membrane and are of major importance to overall energetic efficiency. *ND5* and *ND6* are subunits of the enzyme complex NADH dehydrogenase (ubiquinone) or complex I of the ETC. *CYTB* is the main subunit of coenzyme Q: cytochrome c–oxidoreductase or complex III of the ETC. *COX1* is the main subunit of cytochrome c oxidase or otherwise known as complex IV of the ETC and *COX3 is* a transmembrane subunit of this same complex. Previous transcriptomic experiments have reported a relationship between DEG related to the complexes of ETC and RFI [181, 247]. For example, increased levels of *COXII* (complex IV) and NADH dehydrogenase subunits have been shown to be associated with efficient animals at the protein and transcriptome level [181, 225, 238, 247, 322] in more efficient animals. However, to the best of the author's knowledge, this is the first report of an up-regulation of *COX1, ND5, ND6, CYTB* and *COX3* in skeletal muscle tissue of cattle of low vs high RFI.

Using GO enrichment analysis through the DAVID platform, we identified important processes underlying feed efficiency variation in skeletal muscle of heifers. The most enriched cluster contained a plethora of functions related to mitochondrial metabolism, in particular oxidative phosphorylation. Similarly, the top canonical pathways identified by IPA were mitochondrial function and oxidative phosphorylation. Taken together, these analyses highlight a greater

capacity for mitochondrial function in the low RFI animals. The relationship between mitochondria and RFI has been addressed previously and mitochondrial dysfunction and oxidative stress have been implicated as contributing to variation in feed efficiency [114, 181, 229, 230, 247, 302]. Mitochondria are highly dynamic organelles that are responsible for 90% of the energy production in the body and are major reactive oxygen species (ROS) regulators [323]. It seems likely that variation in mitochondrial function could contribute to variation in energy utilisation. Furthermore, it has been demonstrated that feed efficient animals exhibit greater capacity to modulate conditions of oxidative stress [287].

Feed efficient animals have been shown to have a higher activity of all enzymes of the ETC across multiple species [229, 230, 302] and the present study was in agreement with this. The DEG in the current study encode proteins involved in Complexes I, III and IV of the ETC indicating an impaired oxidative phosphorylation system in the skeletal muscle of the less efficient heifers. These results complement previous research suggesting an association between decreased respiration capacity and increased ROS production in less efficient animals [229, 324]. Moreover, the results of the present study are reinforced by the observation of Kong et al. (2016) in which the mitoproteome was skewed towards high FE birds despite no difference in mitochondrial DNA between phenotypes, suggesting an increase in mitochondrial activity in the high feed efficiency phenotype [322].

We identified 11 DEGs in the hepatic tissue of high and low RFI bulls. Eight of these genes were upregulated in the low RFI animals. These included: *HSPA5 (78 kDa glucose –regulated protein precursor), CRELD2 (cysteine rich with EGF like domains 2), HYOU1 (hypoxia up-regulated protein 1 precursor), MANF (mesencephalic astrocyte derived neurotrophic factor), ACTA2 (actin, alpha2, smooth skeletal muscle, aorta), ENSBTAG00000003151 (DNAJ heatshock protein family (Hsp40) member B11, GMPPB (GDP-mannose pyrophosphorylase B), GNPNAT1 (glucosamine-phosphate N-acetyltransferase 1).* Three genes were downregulated in the low RFI bulls. These included; *LIMS2 (LIM zinc finger domain containing*

2), GSTTP1 (glutathione S-transferase theta pseudogene 1), DBP (D-box binding PAR bzip transcription factor). HSPA5, CRELD2, HYOU1, ACTA2, ENSBTA00000003151, and *GSTTP1* have previously been implicated in variation in feed efficiency [181, 279, 287, 301, 316, 318, 325-329] and potentially are candidate biomarkers for this complex trait.

GO enrichment using DAVID revealed one enriched cluster in the upregulated DEG from the low RFI bulls, which included signalling functions, disulphide bonding and peptide signalling. IPA identified Aldosterone Signalling in Epithelial cells, GDP-mannose Biosynthesis, NRF2 mediated Oxidative Stress Response and EIF2 Signalling as the top canonical pathways related to RFI in bull hepatic tissue. The results of the GO enrichment and IPA analysis indicate that oxidative response, protein mediation and cell signalling in the liver are likely to be processes that are influencing variation in feed efficiency.

The aldosterone signalling pathway was identified by IPA as the top canonical pathway due to the upregulation of the genes *HSPA5* and *DNAJB11* in the hepatic tissue of low RFI bulls. Aldosterone is secreted by the adrenal glands and has a major role in electrolyte and fluid homeostasis. The aldosterone signalling pathway has been implicated in feed efficiency previously in the spleen of inefficient animals [319] and, interestingly, a GWAS study with cattle identified this pathway to be associated with variation in feed conversion ratio [330]. The protein encoded by *HSPA5* is a member of the *HSP70* family and as this protein interacts with many endoplasmic reticulum (ER) proteins it is likely to be a major player in monitoring protein transport through the cell [331]. *DNAJB11* is involved in protein processing and metabolism of proteins and serves as a co-chaperone for *HSPA5* in the ER [331]. One of the many functions of this family of proteins is to stabilize new proteins by ensuring correct folding or by helping refold proteins that have already been damaged by cell stress. Both *DNAJB11* and *HSPA5* have been implicated with feed efficiency previously [287, 296, 316, 319, 332]. In agreement with the current study, other recent work from our group (Keogh et al., 2016) observed that animals undergoing compensatory growth with concomitant improvements in feed efficiency have a

higher hepatic transcript abundance of *DNAJB11* and *HSPA5* [296]. Similarly, an upregulation of *HSPA5* was observed in the hepatic tissue of low RFI animals by Paradis et al. (2015) [301]. Taken together these results are indicative of a greater capacity in controlling cellular function and organisation as well as protein metabolism in more feed efficient animals.

NRF2 mediated oxidative stress response was observed to be an overrepresented pathway in the hepatic tissue of high RFI bulls in the current study due to the upregulation of the genes *GSTT1* and *DBP*. NRF2 is a member of the cap 'n' collar basic region leucine zipper (cnc bZip) group of transcription factors [333]. This transcription factor is ubiquitously expressed in tissues but is only activated in response to a range of oxidative and electrophilic stimuli including ROS, antioxidants, glucose induced oxidative damage, heavy metals, and certain disease processes [333-335]. This canonical pathway has been associated with feed efficiency in a number of studies [279, 317, 329, 336, 337]. *GSTT1* is a member of the glutathione *S*-transferase family and is involved in metabolism of xenobiotics and in catalysing reactions between the antioxidant glutathione and a host of potentially toxic compounds, highlighting it as an important homeostatic molecule [338]. The glutathione S-transferase family has been implicated in FE previously [279, 317, 329, 332, 337]. In agreement with the current study Chen et al. (2011) and Lindholm-Perry et al. (2014) observed an upregulation of these genes in feed inefficient cattle [317, 329]. *DBP* is a Protein Coding gene and amongst its cited functions is activation of circadian gene expression. GO annotations related to this gene include transcription factor activity, sequence-specific DNA binding and transcriptional activator activity and RNA polymerase II core promoter proximal region sequence-specific binding [339]. While *DBP* has never been associated in the published literature, with feed efficiency in cattle before now, it has been shown that mice with increased FE have a lower expression of *DBP* [340] which is in agreement with the present study. Taken together, our work and that of the aforementioned authors, suggest that less efficient animals are exhibiting an increased oxidative stress, reflected in their increased anti-oxidation activities.

The differential expression of *HYOU1*, *CRELD2*, and *ACTA2* in the present study are also noteworthy as all three genes have been previously observed as differentially expressed in relation to feed efficiency in cattle [287, 296, 341]. While the biological significance of these genes in relation to feed efficiency remains unclear, due to their consistent presence in the literature, they should not be ruled out as potential biomarkers for this trait.

The present study contributes to the published knowledge base regarding the transcriptomic regulation of variation in feed efficiency. Our work, in combination with that of others as previously mentioned, highlights common genes underpinning feed efficiency in cattle, as measured by RFI, regardless of breed or genetic background. RNA-seq analysis is an exploratory approach that provides new hypotheses to be further investigated by other complementary approaches including global proteomics and ultimately, potential variation in the genes identified in this study may provide a basis for the selection of candidate biomarkers for the RFI trait and, following appropriate validation, contribute to genomic selection breeding programmes to improve feed efficiency in beef cattle. However, the key message from this work highlights the inconsistency in gene expression profiles across genders of the same genetic merit. This is a worrying prospect for the identification of robust and accurate biomarkers for RFI with widespread utility. While this inconsistency may be explained by the differences in physiological maturity of the two genders, it indicates that extensive further investigation is needed before biomarker selection for RFI can be adopted.

Chapter 6

CHAPTER 6. THE EFFECT OF BREED AND DIET ON THE SKELETAL MUSCLE TRANSCRIPTOME OF BEEF CATTLE DIVERGENT FOR RESIDUAL FEED INTAKE

6.1 Introduction

Feed accounts for up to 75% of the variable costs in beef cattle production systems [342] thus having a fundamental influence on the economic sustainability of the enterprise. Improving FE has the potential to reduce feed costs while maintaining economically favourable traits such as growth and muscle gain [343]. Residual feed intake is a measure of feed efficiency defined as the difference between an animal's actual versus its predicted feed intake [25]. For example, animals with low RFI (efficient) status typically consume between 10% to 20% less feed than their high RFI (inefficient) counterparts for the same level of performance [26, 27, 58, 62, 83, 95, 247]. RFI is an attractive trait for selection in breeding programmes due to its moderate heritability [22, 37, 46, 344], and the weight of published evidence indicates that the trait is not, in the main, antagonistically correlated with other economically relevant traits at either a genetic or phenotypic level [22, 44].

The main impediment to genetic progress and adoption of selection for feed efficiency is the logistics and expense of measuring individual animal intake necessary to accurately identify feed efficient animals. Consequently, the development of predictive biological markers that can be used to accurately select cattle with improved feed efficiency potential, is an attractive alternative to direct measurement [345]. An additional concern for forage based beef production systems, such as that practiced in Ireland, is the potential for a genotype x environment interaction manifested as inconsistent feed efficiency and animal performance ranking across different diet types. For example, in most regions worldwide, prime cattle are reared at pasture up to weaning. However, there is significant variation thereafter in backgrounding and finishing regimen depending on the relative cost of forage and concentrate feeds at the target age at slaughter. Variation in energy density and intake capacity characteristics of feedstuff impacts

on feed efficiency potential. Moreover, although relatively repeatable, we and others have reported re-ranking of cattle on RFI when offered either the same diet [26] or diets differing in chemical composition/energy density [346] over successive feed intake recording periods [58, 347]. This is strongly indicative of a genotype x environment interaction for feed efficiency. Indeed, breeding values for feed efficiency within the national breeding programme in Ireland and other countries are based on animals consuming a high-energy diet, however, the vast majority of commercially produced cattle in Ireland are produced on grass-based diets. Furthermore, it is well established that different breeds have different inherent feed efficiency capacity [12]. In light of this, research to elucidate if a genotype x environment interaction exists, is warranted in order to inform future breeding programmes [343].

Genomic technologies offer the tools to elucidate the genetic architecture of economically important traits such as RFI with a view to informing industry deployment of DNA based biomarkers. It follows that genomic investigations of this complex trait should include the influence of environmental factors such as diet, age and breed. Transcriptomic analysis can provide evidence to focus on the critical genes and regulatory elements likely to affect phenotype [348, 349]. It follows that genes consistently differentially expressed across diet and breed with regard to RFI are likely to be robust candidates for further interrogation or indeed biomarker selection. It is imperative to the future sustainability of the Irish beef cattle industry to confirm that cattle reared on, and selected under a high concentrate regime will be the same animals that sire more efficient progeny reared predominantly on pasture-based systems. There has been little to no agreement between studies that have utilised global transcriptomic or GWAS approaches in terms of identifying common processes associated with variation in RFI, particularly across nutritional conditions, age and breed. Therefore, further information is required on the influence of a genotype x environment interaction on differential gene expression profiles for the feed efficiency trait.

Skeletal muscle is a highly metabolic organ accounting for up to 50% of body mass of cattle[297]. In addition, different cattle breeds differ in skeletal muscle gene expression profiles [350], therefore, it is logical that variation in the metabolism of this organ could influence feed efficiency status. Thus, the aim of this study was to characterise the global transcriptome of skeletal muscle tissue in order to elucidate the molecular mechanisms underpinning RFI in muscle of beef cattle and whether this is consistent across (i) contrasting breeds and (ii) dietary regimes. Such information could be harnessed, to select key genes that could be further interrogated for the presences of genetic variants that may regulate the expression of RFI in beef cattle. Such information following validation will facilitate the selection of animals with enhanced feed efficiency and is implementable through genomic selection breeding programmes ongoing in Ireland and elsewhere.

6.2 Results

6.2.1 Animal Model

Descriptive statistics are presented separately for both breed types across the three dietary phases in Tables 6.1-6.3, respectively. For both breed types, in all three phases, mean RFI values were, by design, close to zero. The effect of RFI status and diet on DMI, RFI and performance is presented in Tables 6.1-6.3. In the first high concentrate phase (H1), high RFI CH steers consumed 16% more, and high RFI HF steers consumed 12% more, than their low RFI counterparts, respectively (P<0.001; Table 6.1). In the zero-grazed grass phase (ZG), high RFI CH steers consumed 8% more, and high RFI HF steers consumed 11% more, than their low RFI counterparts, respectively (P<0.001; Table 6.2). In the final high concentrate phase (H2), high RFI CH steers consumed 15% more, and high RFI HF steers consumed 17% more, than their low RFI counterparts (P<0.001; Table 6.3). In each dietary phase within breed, mid-test MBW and ADG did not differ (P>0.05) between the two contrasting RFI groups.

Table 6.1. Feed intake, RFI and growth traits for CH and HF steers ranked low and high RFI offered a high concentrate diet at yearling stage (H1).

Trait	**Breed**	Low	High	s.e	*P*-value
DMI (kg/d)	CH	7.8	9	0.11	<0.001
	HF	8.3	9.3	0.15	<0.001
RFI (kg/d)	CH	-0.5	0.56	0.04	<0.001
	HF	-0.5	0.53	0.047	<0.001
ADG (kg/d)	CH	1.3	1.4	0.05	0.43
	HF	1.4	1.4	0.04	0.78
MBW (kg/d)	CH	95	96	1.1	0.79
	HF	81	80	1.7	0.9

DMI = Dry Matter Intake ; RFI = Residual feed intake; ADG = Average Daily Gain; MBW = metabolic body weight; CH = Charolais; HF = Holstein Friesan; Low = Low RFI; High = High RFI; s.e = standard error of the mean.

Table 6.2. Feed intake, RFI and growth traits for CH and HF steers ranked low and high RFI offered a zero grazed grass diet at growing stage (ZG).

Trait	Breed	Low	High	s.e	*P*-value
DMI (kg/d)	CH	8.8	9.4	0.08	<0.001
	HF	9.1	10	0.11	<0.001
RFI (kg/d)	CH	-0.35	0.35	0.026	<0.001
	HF	-0.42	0.56	0.048	<0.001
ADG (kg/d)	CH	1.4	1.4	0.03	0.96
	HF	1.2	1.3	0.04	0.85
MBW (kg/d)	CH	96	97	1.1	0.8
	HF	82	80	1.7	0.9

DMI = Dry Matter Intake ; RFI = Residual feed intake; ADG = Average Daily Gain; MBW = metabolic body weight; CH = Charolais; HF = Holstein Friesan; Low = Low RFI; High = High RFI; s.e = standard error of the mean.

Table 6.3. Feed intake, RFI and growth traits for CH and HF steers ranked low and high RFI offered a high concentrate diet at finishing stage (H2).

Trait	**Breed**	Low	High	s.e	*P*-value
DMI (kg/d)	CH	10.8	12.3	0.16	<0.001
	HF	11.6	13.6	0.2	<0.001
RFI (kg/d)	CH	-0.75	0.76	0.058	<0.001
	HF	-1.01	1.03	0.086	<0.001
ADG (kg/d)	CH	1.4	1.4	0.05	0.64
	HF	1.3	1.3	0.07	0.98
MBW (kg/d)	CH	96	97	1.1	0.79
	HF	82	81	1.5	0.9

DMI = Dry Matter Intake ; RFI = Residual feed intake; ADG = Average Daily Gain; MBW = metabolic body weight; CH = Charolais; HF = Holstein Friesan; Low = Low RFI; High = High RFI; s.e = standard error of the mean.

6.2.2 RNA-seq data analysis

6.2.2.1 Input, preprocessing, read mapping and quantification

Due to poor RNA quality and sample loss, some samples were excluded from the study prior to cDNA synthesis. In total, muscle cDNA from CH (n=52) and HF (n=58) was successfully sequenced with an average of 21.7 million reads per sample. Details of the number of samples used for each comparison can be seen in Table 6.4. Effects of read-filtering, adapter trimming and read mapping are presented in Appendix Table 9.2. Briefly, an average of 21.7 million reads per sample was obtained with an average phred (measure of the quality of the identification of the nucleobases generated by automated DNA sequencing) score of 98 % and GC content of 51 %. An average of 90 % of reads across all samples mapped uniquely to the bovine reference genome.

6.2.2.2 Expression analysis

Figures 6.1-6.4 present a visual analysis of the VST transformed gene expression data for all input libraries. Figure 6.1 illustrates the smoothed distribution of all data as a probability density function. Figure 6.2 illustrates the pairwise correlation coefficients of each input library. Figure 6.3 provides a hierarchical clustering analysis of the expression data. Figure 6.4 gives a visual representation of the PCA analysis results. Collectively, these figures illustrate the overall distribution of expression values across all libraries/conditions, and the results of unsupervised clustering analysis.

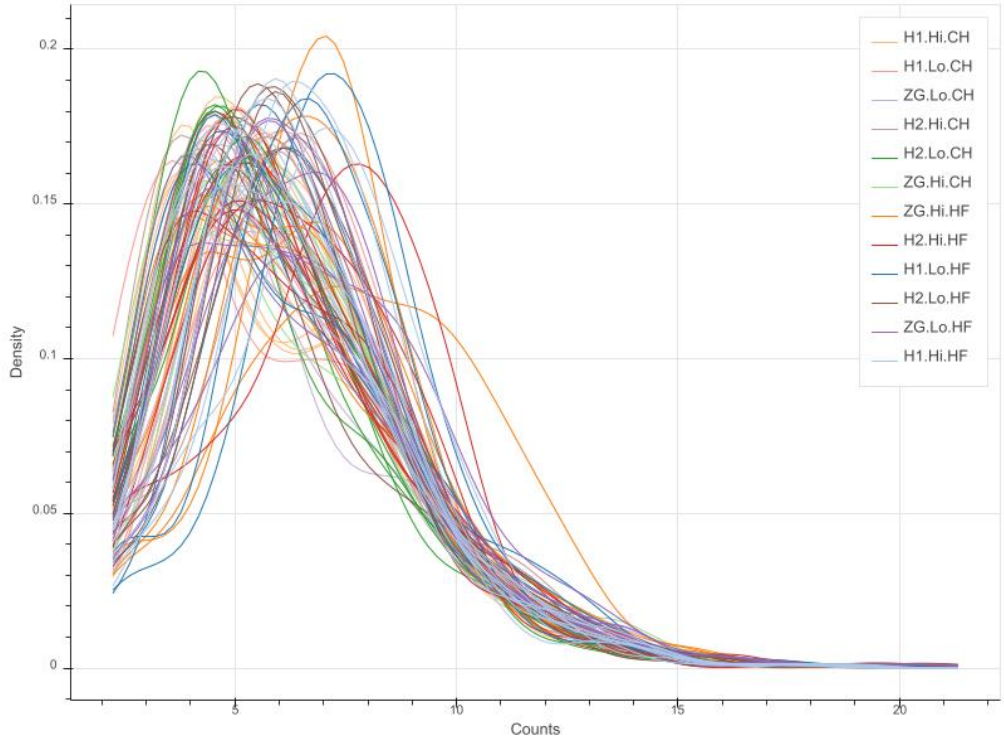

Figure 6.1. Kernel Density Estimation displaying gene expression patterns of all input libraries in the study. H1 = high concentrate, phase 1; ZG = zero grazed grass, phase 2, H2 = high concentrate, phase 3; Hi = High RFI; Lo = Low RFI; CH= Charolais; HF = Holstein Friesian.

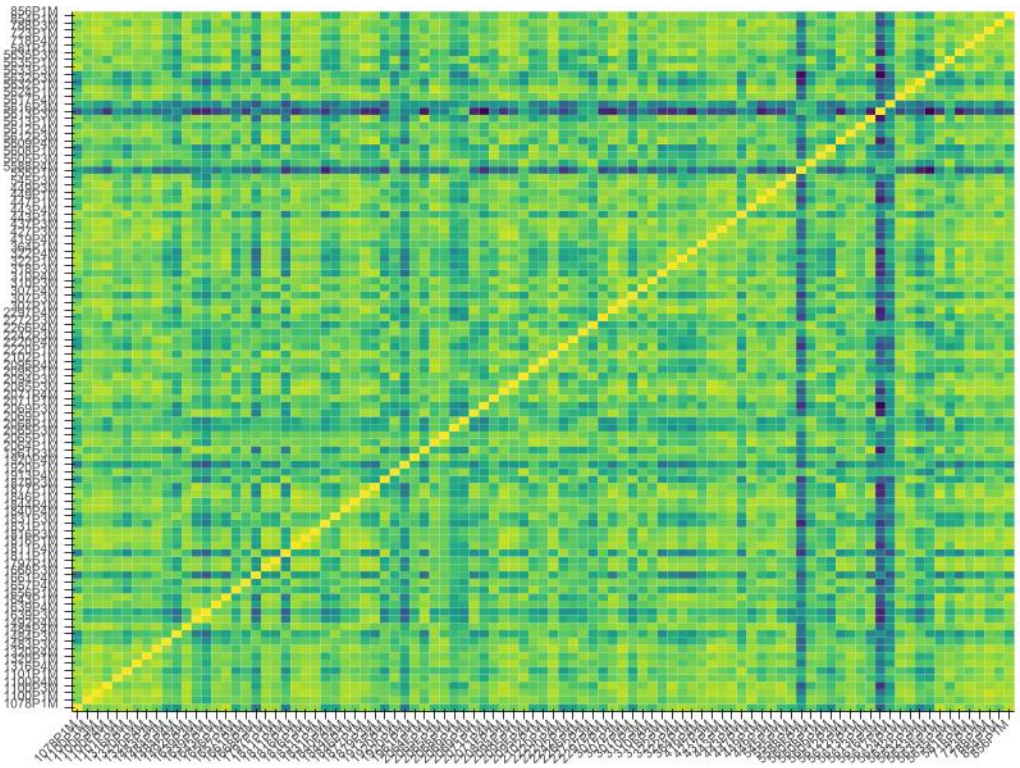

Figure 6.2. Correlation Analysis of all input libraries in the study. Yellow squares indicate samples that are highly correlated with each other. H1 = high concentrate, phase 1; ZG = zero grazed grass, phase 2, H2 = high concentrate, phase 3; Hi = High RFI; Lo = Low RFI; CH= Charolais; HF = Holstein Friesian.

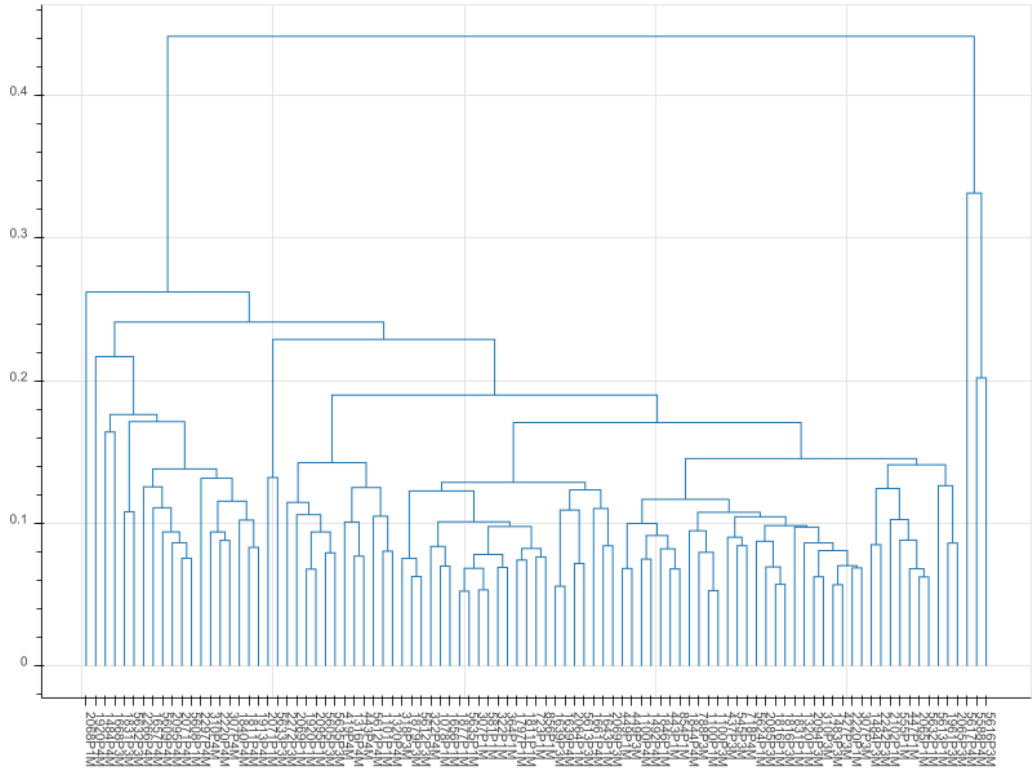

Figure 6.3. Hierarchical cluster analysis of all input libraries in the study depicting the gene expression relationships between all input samples. H1 = high concentrate, phase 1; ZG = zero grazed grass, phase 2, H2 = high concentrate, phase 3; Hi = High RFI; Lo = Low RFI; CH= Charolais; HF = Holstein Friesan.

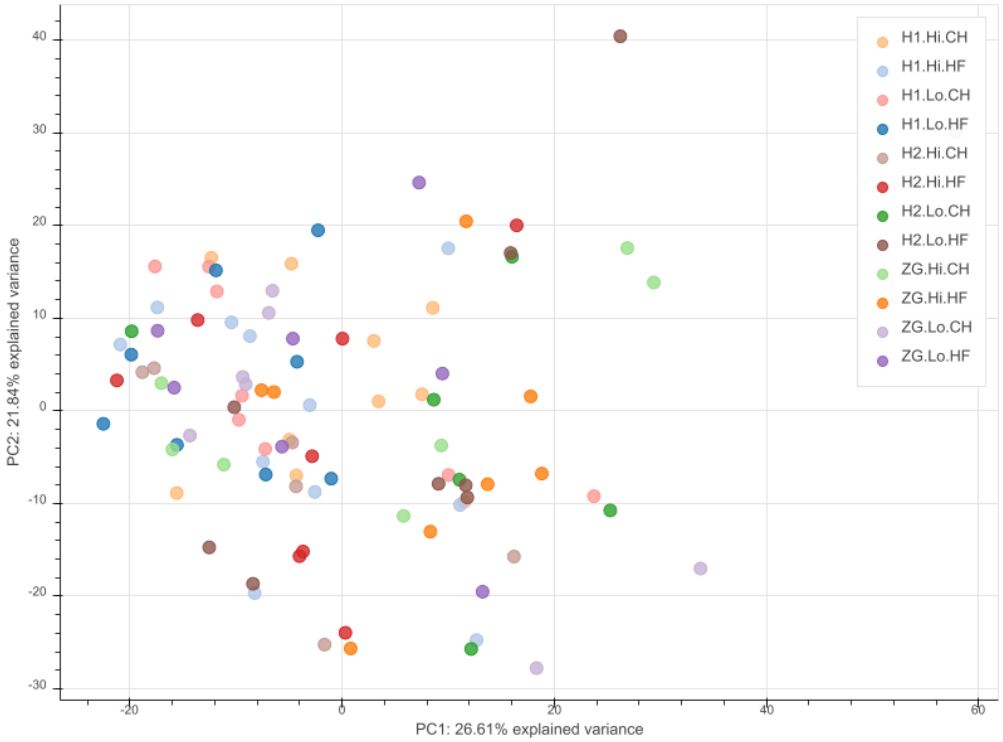

Figure 6.4. Principal components analysis of all input libraries in the study. H1 = high concentrate, phase 1; ZG = zero grazed grass, phase 2, H2 = high concentrate, phase 3; Hi = High RFI; Lo = Low RFI; CH= Charolais; HF = Holstein Friesian.

6.2.2.3 Pair-wise contrast analysis

The results of all pair-wise tests are summarised in Table 6.4. This Table outlines the names of base and test sub-groups, the number of libraries included in each sub-group, and the number of genes found to be differentially expressed between them. Figures 6.5-6.7 present a visual representation of the PCA analysis for the CH animals under all feeding conditions. There were no differentially expressed genes between high and low RFI CH steers when offered either high concentrate or zero-grazed grass during their growing phase. However when offered a high concentrate diet during the finishing phase, 4 genes were differentially expressed between high and low RFI CH steers. Figures 6.8-6.10 present a visual representation of the PCA analysis for the HF steers under all feeding conditions. There were no differentially expressed genes between high and low HF steers offered a high concentrate diet in either the growing or finishing phases. However, when the animals were offered zero-grazed grass in the growing phase there was differential expression of 170 genes between the high and low phenotypes.

Table 6. 4. Summary of pair-wise differential expression test

Base	Test	#base	#test	#gene
H1.Lo.CH	H1.Hi.CH	8	10	0
H1.Lo.HF	H1.Hi.HF	8	11	0
H2.Lo.CH	H2.Hi.CH	6	6	4
H2.Lo.HF	H2.Hi.HF	8	8	0
ZG.Lo.CH	ZG.Hi.CH	7	7	0
ZG.Lo.HF	ZG.Hi.HF	8	8	170
H1.Hi.CH	H2.Hi.CH	10	6	122
H1.Lo.CH	H2.Lo.CH	8	6	2
H2.Lo.CH	H2.Hi.CH	6	6	4
ZG.Hi.CH	H1.Hi.CH	7	10	12
ZG.Hi.CH	H2.Hi.CH	7	6	54
ZG.Lo.CH	H2.Lo.CH	7	6	28
ZG.Lo.CH	H1.Lo.CH	7	8	11
H1.Hi.HF	H2.Hi.HF	11	8	2098
H1.Lo.HF	H2.Lo.HF	8	8	693
ZG.Hi.HF	H1.Hi.HF	8	11	330
ZG.Hi.HF	H2.Hi.HF	8	8	513
ZG.Lo.HF	H2.Lo.HF	8	8	488
ZG.Lo.HF	H1.Lo.HF	8	8	30
H1.Hi.HF	H1.Hi.CH	11	10	15
H1.Lo.HF	H1.Lo.CH	8	8	1
H2.Hi.HF	H2.Hi.CH	8	6	55
H2.Lo.HF	H2.Lo.CH	8	6	45
ZG.Hi.HF	ZG.Hi.CH	8	7	635
ZG.Lo.HF	ZG.Lo.CH	8	7	53

Contrasted sub-groups are given by *base* and *test* in which the gene expression of the *test* sub-group was tested against that of the *base* sub-group. *#base* and *#test* describes number of libraries included in each sub-group. *#gene* gives the number of genes found to be differentially expressed (adjusted p-value <0.1). H1 = high concentrate, phase 1; ZG = zero grazed grass, phase 2, H2 = high concentrate, phase 2; Hi = High RFI; Lo = Low RFI

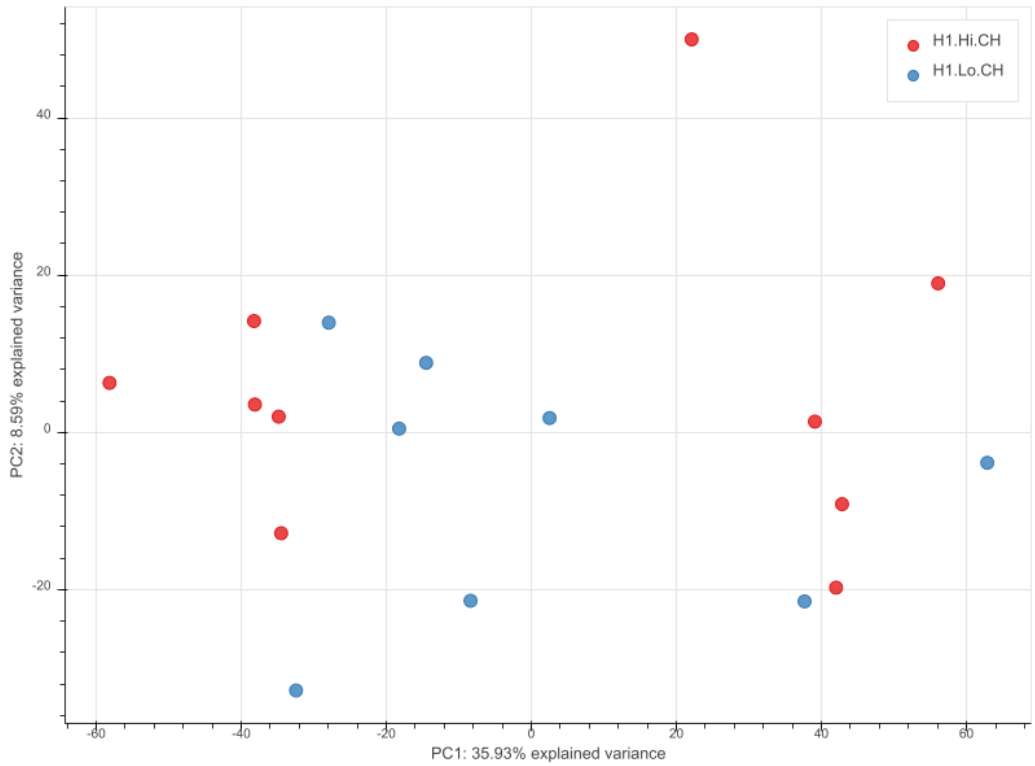

Figure 6. 5 . Principal components analysis of all contrasted libraries in CH cattle in phase 1. H1 = high concentrate, phase 1; Hi = High RFI; Lo = Low RFI; CH= Charolais.

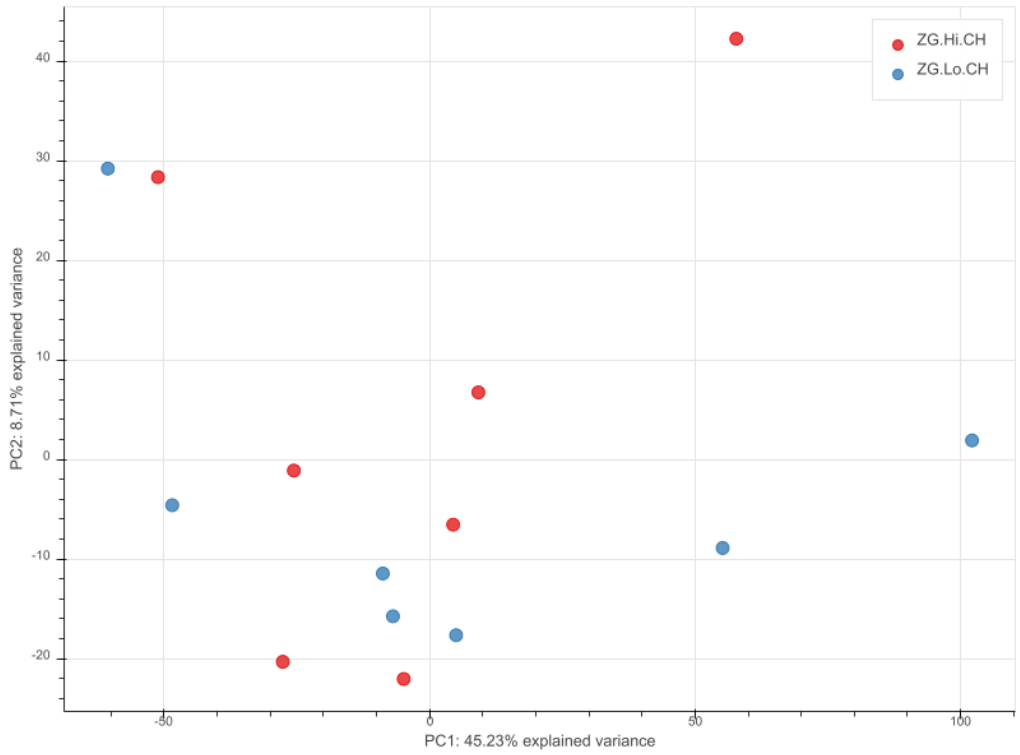

Figure 6.6. Principal components analysis of all contrasted libraries in CH cattle in phase 2. ZG = zero grazed grass, phase 3; Hi = High RFI; Lo = Low RFI; CH= Charolais.

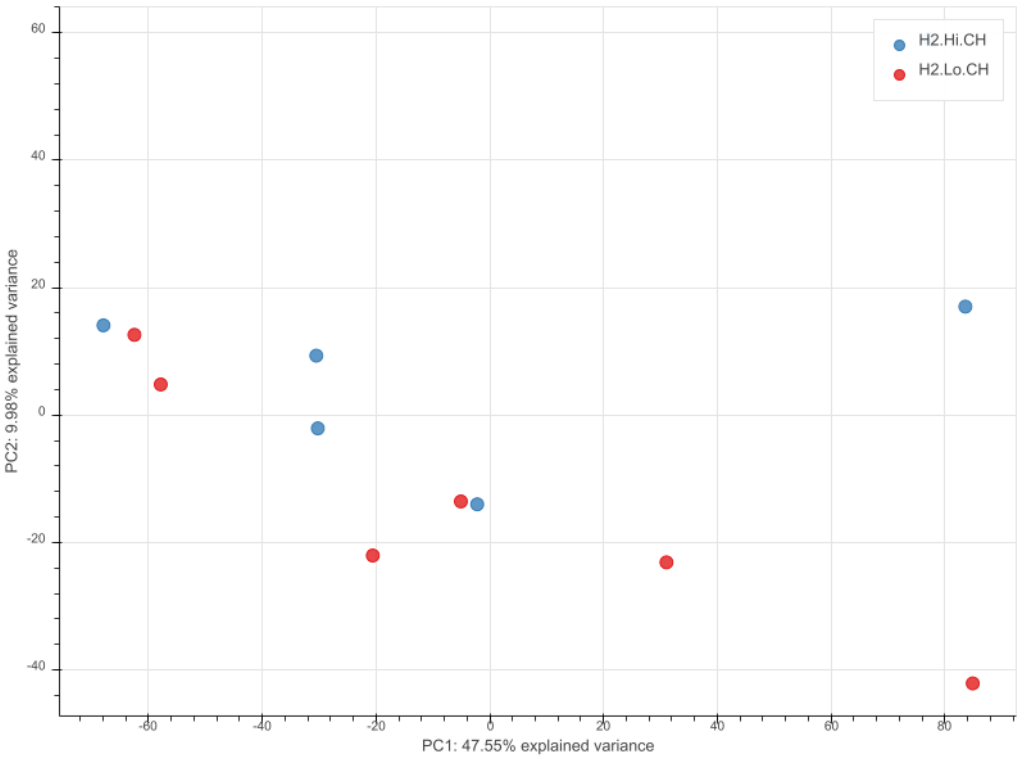

Figure 6.7 . Principal components analysis of all contrasted libraries in CH cattle in phase 3. H2 = high concentrate, phase 3; Hi = High RFI; Lo = Low RFI; CH= Charolais.

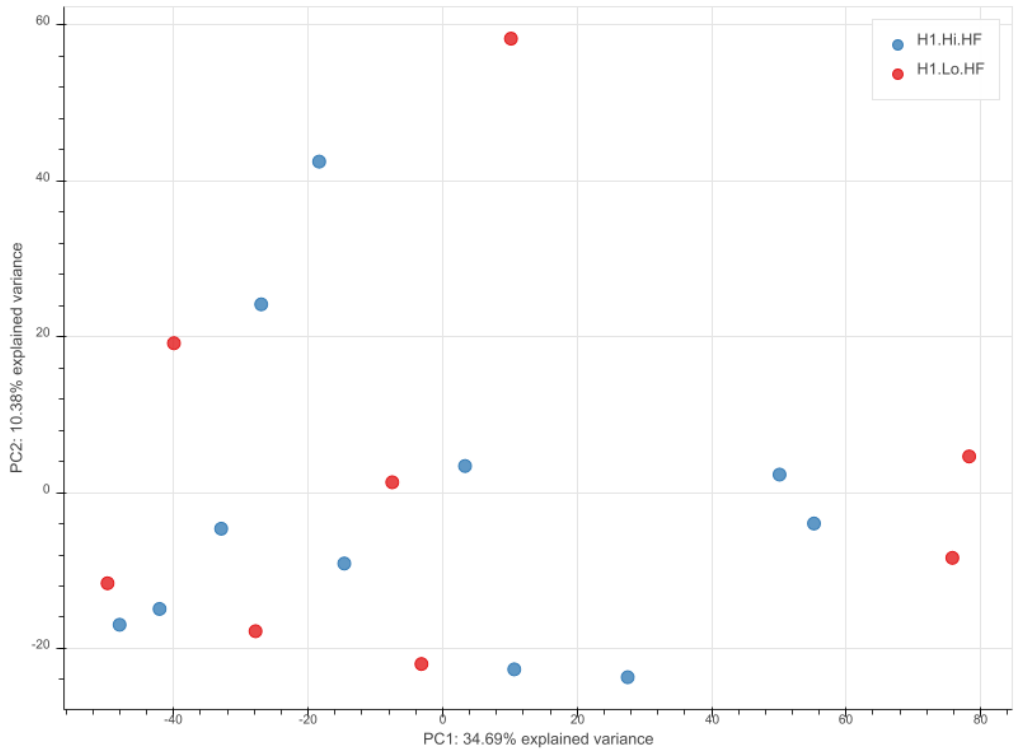

Figure 6.8. Principal components analysis of all contrasted libraries in HF cattle in phase 1.H1 = high concentrate, phase 1Hi = High RFI; Lo = Low RFI; HF = Holstein Friesian.

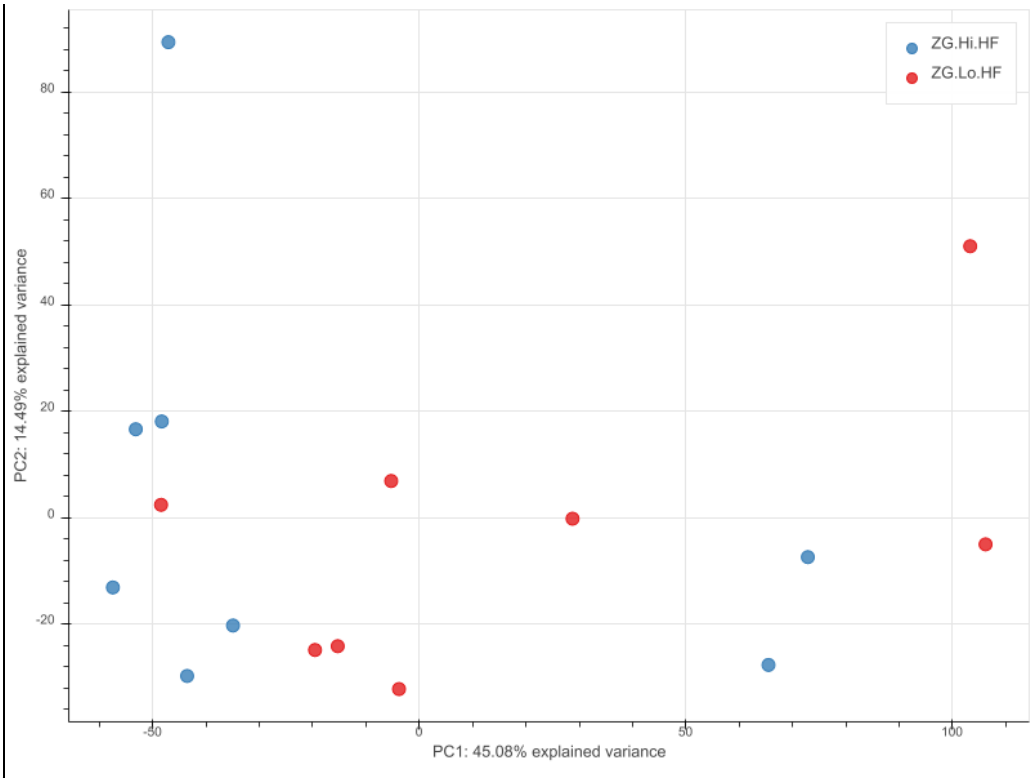

Figure 6.9. Principal components analysis of all contrasted libraries in HF cattle in phase 2. ZG = zero grazed grass, phase 2; Hi = High RFI; Lo = Low RFI; HF = Holstein Friesan.

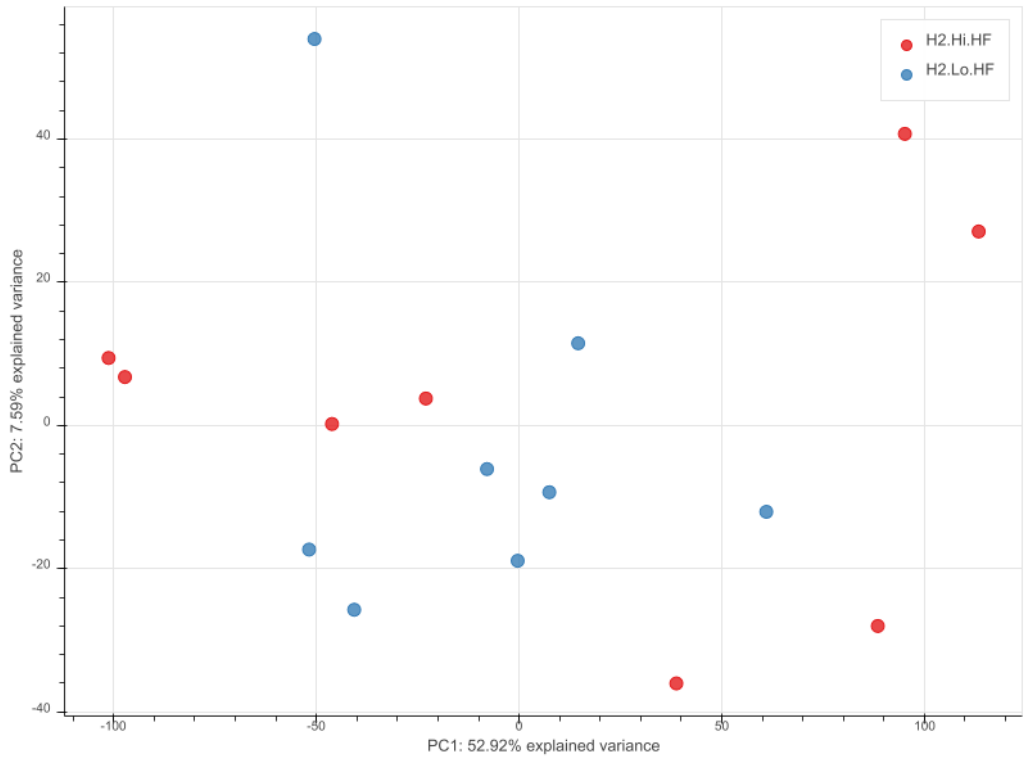

Figure 6.10. Principal components analysis of all contrasted libraries in HF cattle in phase 3. H2 = high concentrate, phase 3; Hi = High RFI; Lo = Low RFI; HF = Holstein Friesian.

6.2.2.4 Differential expression analysis

Details of DEG are presented in Tables 6.5 and 6.6, while Figures 6.11 and 6.12 illustrate the differences in gene expression as volcano plots. For HF animals offered a zero grazed grass diet during the growing phase, a total of 170 genes were found to be differentially expressed between high and low RFI animals, with an adjusted p-value of 0.1, details of which are presented in Table 6.5. For CH animals, a total of 4 genes were found to be differentially expressed between high and low RFI animals offered a high concentrate diet during the finishing phase., with an adjusted p-value of 0.1, details of which are presented in Table 6.6. These genes were *keratin 75(KRT75), 7SK, nuclear RNase P(RNaseP nuc)* and *Y RNA.*

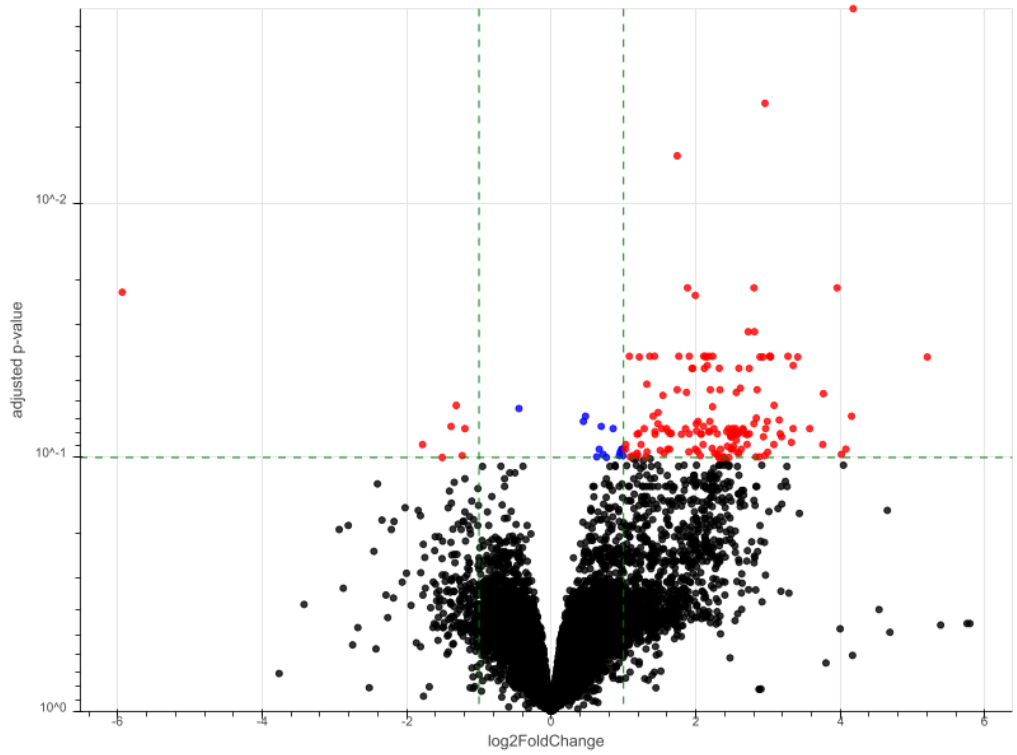

Figure 6.11. Volcano plot depicting an overview of differential gene expression in HF animals divergent for RFI in phase 2 (zero grazed grass). The red dots indicate points-of-interest that display both large magnitude fold-changes (x axis) and high statistical significance (-log10 of p value, y axis). The dashed horizontal line shows where p = 0.1 with points above the line having $p < 0.1$ and points below the line having $p > 0.1$.

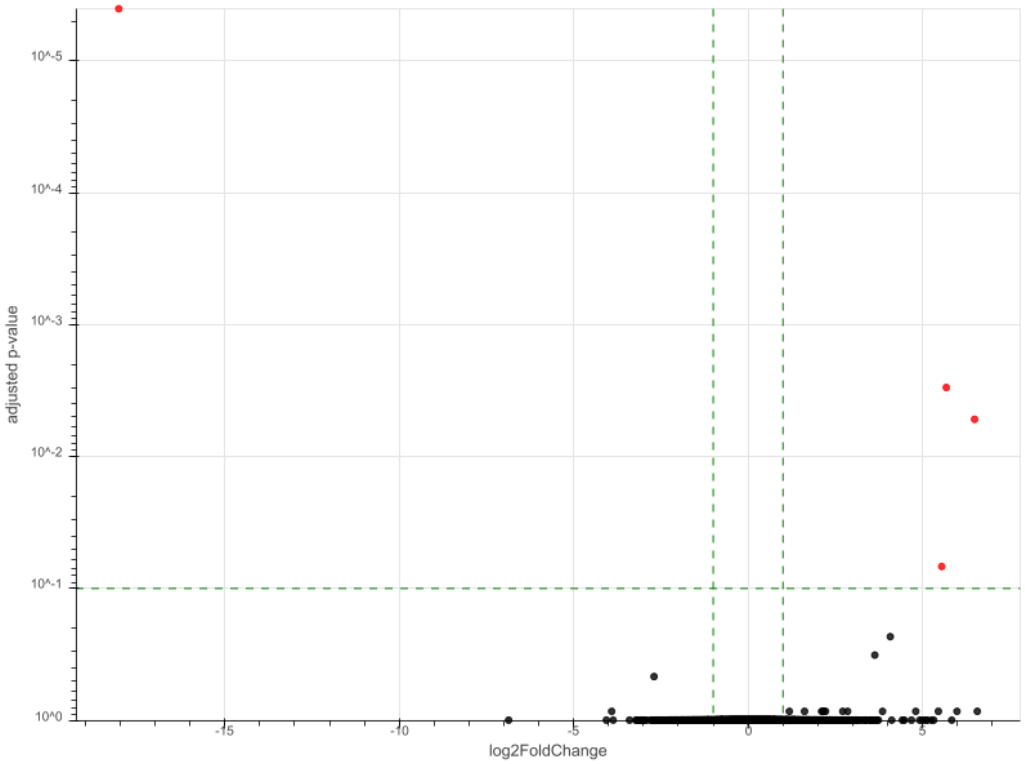

Figure 6.12. Volcano plot depicting an overview of differential gene expression in CH animals divergent for RFI in phase 3 (high concentrate 2). The red dots indicate points-of-interest that display both large magnitude fold-changes (x axis) and high statistical significance (-log10 of p value, y axis). The dashed horizontal line shows where p = 0.1 with points above the line having $p < 0.1$ and points below the line having $p > 0.1$.

6.2.2.5 Pathway analysis

Unfortunately, only one pair-wise contrast, high and low RFI HF steers offered zero grazed grass, was suitable for pathway analysis. Functional analysis using both the up- and down-regulated genes was performed using DAVID software and results are presented in Table 6.7. The top enriched functions were related to signalling, innate immunity, receptor binding and metabolite synthesis and transport. DEG were successfully mapped to a molecular or biological pathway and/or category in the IPA database. DEG were analysed and separated according to their biological function within IPA. The top canonical pathways affected by RFI in animals consuming zero grazed grass are presented in Table 6.8. These were IL-6 signalling, role of pattern recognition receptors in recognition of bacteria and viruses, NF-B signalling, serotonin and melatonin biosynthesis and role of osteoblasts, osteoclasts and chondrocytes in rheumatoid arthritis. One network, organismal injury and abnormalities, gastrointestinal disease and inflammatory disease was identified to be associated with RFI.

Table 6.5 Differentially expressed genes in muscle between HF animals divergent for RFI when offered a zero grazed grass diet

	log2FC	pvalue	padj	Symbol	Name
ENSBTAG00000004602	4.17909	1.16E-07	0.001701	PITX1	paired like homeodomain 1
ENSBTAG00000021287	2.95815	5.45E-07	0.004014	SLC16A7	solute carrier family 16 member 7
ENSBTAG00000001822	1.74305	1.32E-06	0.006464	SNX10	sorting nexin 10
ENSBTAG00000015330	2.8058	5.83E-06	0.02144		
ENSBTAG00000039035	3.95726	8.74E-06	0.02144	HSPA6	heat shock protein family A (Hsp70) member 6
ENSBTAG00000021263	1.88523	8.64E-06	0.02144	CYP4V2	cytochrome P450 family 4 subfamily V member 2
ENSBTAG00000016063	-5.938	1.06E-05	0.022309	DNER	delta/notch like EGF repeat containing
ENSBTAG00000006307	1.99406	1.25E-05	0.022964	HSD17B11	hydroxysteroid 17-beta dehydrogenase 11
ENSBTAG00000017722	2.8135	2.17E-05	0.03192	F5	coagulation factor V
ENSBTAG00000015654	2.72656	2.00E-05	0.03192	PON1	paraoxonase 1
ENSBTAG00000020554	1.42897	2.98E-05	0.039855	AIF1	allograft inflammatory factor 1
ENSBTAG00000016177	2.18405	3.34E-05	0.039887	FGL1	fibrinogen like 1
ENSBTAG00000015290	2.10859	5.54E-05	0.039887	BAIAP2L2	BAI1 associated protein 2 like 2
ENSBTAG00000014197	2.13262	5.41E-05	0.039887	SLC38A4	solute carrier family 38 member 4
ENSBTAG00000047040	3.27615	4.95E-05	0.039887		
ENSBTAG00000001638	3.03303	4.23E-05	0.039887		
ENSBTAG00000039995	1.91068	4.43E-05	0.039887		complement factor H precursor
ENSBTAG00000048195	1.36281	5.96E-05	0.039887	PIGX	phosphatidylinositol glycan anchor biosynthesis class X
ENSBTAG00000012779	1.08099	4.92E-05	0.039887	FAM49A	family with sequence similarity 49 member A

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6.5 (contd). Differentially expressed genes in muscle between HF animals divergent for RFI when offered a zero grazed grass diet

	log2FoldChange	pvalue	padj	Symbol	Name
ENSBTAG00000025942	1.76477	4.46E-05	0.039887	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)
ENSBTAG00000000897	2.23496	5.88E-05	0.039887	IQGAP2	IQ motif containing GTPase activating protein 2
ENSBTAG00000013907	3.03409	7.08E-05	0.040146	CRP	C-reactive protein
ENSBTAG00000020715	5.20522	7.12E-05	0.040146	PIK3C2G	phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 gamma
ENSBTAG00000003196	2.14472	6.38E-05	0.040146	PAPSS2	bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2
ENSBTAG00000007043	3.41196	6.67E-05	0.040146		
ENSBTAG00000046773	2.92913	7.91E-05	0.040146	MCOLN2	mucolipin 2
ENSBTAG00000022120	2.88966	7.90E-05	0.040146	FGB	fibrinogen beta chain
ENSBTAG00000003068	1.21901	7.91E-05	0.040146	MSMO1	methylsterol monooxygenase 1
ENSBTAG00000034883	2.15977	9.13E-05	0.043375	CCR4	C-C motif chemokine receptor 4
ENSBTAG00000013333	3.34789	8.98E-05	0.043375	GYS2	glycogen synthase 2
ENSBTAG00000020602	2.32604	9.92E-05	0.04449	IDO1	indoleamine 2,3-dioxygenase 1
ENSBTAG00000048246	2.59686	9.97E-05	0.04449		
ENSBTAG00000017404	2.11842	0.000106	0.044542	SEC14L2	SEC14 like lipid binding 2
ENSBTAG00000010841	1.95936	0.00011	0.044542	FMO5	flavin containing monooxygenase 5
ENSBTAG00000005596	2.74077	0.000112	0.044542	IGFBP2	insulin like growth factor binding protein 2
ENSBTAG00000033221	1.32273	0.000133	0.051384	CCDC152	coiled-coil domain containing 152
ENSBTAG00000005585	2.61788	0.000141	0.053272	ANXA13	annexin A13
ENSBTAG00000026236	2.84933	0.000158	0.05407	AZGP1	alpha-2-glycoprotein 1, zinc-binding
ENSBTAG00000021190	2.20136	0.000156	0.05407	CDH2	cadherin 2

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6. 5 (contd). Differentially expressed genes in muscle between HF animals divergent for RFI when offered a zero grazed grass diet

	log2FoldChange	pvalue	padj	Symbol	Name
ENSBTAG00000047621	2.33516	0.000151	0.05407		
ENSBTAG00000002096	1.74278	0.000151	0.05407		low affinity immunoglobulin gamma Fc region receptor III isoform 2 precursor
ENSBTAG00000038067	2.55995	0.000169	0.055368		Metallothionein-1A
ENSBTAG00000017863	1.87122	0.000169	0.055368	SRGN	serglycin precursor
ENSBTAG00000001273	3.76629	0.000175	0.056015	NLRP3	NLR family pyrin domain containing 3
ENSBTAG00000045588	1.54612	0.000182	0.056901		
ENSBTAG00000002255	3.08391	0.000207	0.062304	BHMT	betaine--homocysteine S-methyltransferase
ENSBTAG00000011316	-1.315	0.000205	0.062304	ZC3H12A	zinc finger CCCH-type containing 12A
ENSBTAG00000006447	2.23235	0.000214	0.063073	ACSM3	acyl-CoA synthetase medium-chain family member 3
ENSBTAG00000011278	1.47877	0.000235	0.066476	XYLB	Xylulokinase
ENSBTAG00000017077	0.47369	0.000253	0.068718	CTSL2	Cathepsin L1 Cathepsin L1 heavy chain Cathepsin L1 light chain
ENSBTAG00000005586	1.40989	0.000253	0.068718	GATM	glycine amidinotransferase
ENSBTAG00000005343	4.15613	0.000257	0.068718	TPH1	tryptophan hydroxylase 1
ENSBTAG00000004384	2.83798	0.000266	0.069923	APOF	apolipoprotein F
ENSBTAG00000004680	3.15539	0.000275	0.071102	SLC13A5	solute carrier family 13 member 5
ENSBTAG00000022394	2.98954	0.000299	0.072074		Serum amyloid A protein Amyloid protein A
ENSBTAG00000010609	2.1948	0.000292	0.072074	STK31	serine/threonine kinase 31
ENSBTAG00000001522	0.44495	0.000299	0.072074	MTERF3	mitochondrial transcription termination factor 3
ENSBTAG00000005218	2.03175	0.000292	0.072074	PDE3B	phosphodiesterase 3B
ENSBTAG00000018138	1.476	0.000314	0.073585	GK5	Bos taurus glycerol kinase 5 (putative) (GK5), mRNA.
ENSBTAG00000024647	2.00711	0.000317	0.073585		
ENSBTAG00000038171	2.80515	0.00032	0.073585		
ENSBTAG00000045582	0.69167	0.000343	0.07539	ARPC5	actin related protein 2/3 complex subunit 5

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6.5 (contd). Differentially expressed genes in muscle between HF animals divergent for RFI when offered a zero grazed grass diet

	log2FoldChange	pvalue	padj	Symbol	Name
ENSBTAG00000015086	2.10792	0.00034	0.07539	HSD11B1	hydroxysteroid 11-beta dehydrogenase 1
ENSBTAG00000018499	-1.3868	0.000339	0.07539		Annexin A8
ENSBTAG00000001595	3.34699	0.000401	0.076938		
ENSBTAG00000001271	2.50895	0.000364	0.076938	PLG	Plasminogen
ENSBTAG00000017294	2.65345	0.00039	0.076938		
ENSBTAG00000005638	1.85753	0.000367	0.076938	CABYR	calcium binding tyrosine phosphorylation regulated
ENSBTAG00000000070	2.96695	0.000423	0.076938	F13B	coagulation factor XIII B chain
ENSBTAG00000019225	0.85672	0.000363	0.076938	SLC39A14	solute carrier family 39 member 14
ENSBTAG00000005910	2.45193	0.000419	0.076938	LEPR	leptin receptor
ENSBTAG00000023652	1.53182	0.000374	0.076938	PROS1	protein S (alpha)
ENSBTAG00000000258	2.25014	0.000405	0.076938		
ENSBTAG00000009768	1.59537	0.0004	0.076938	IFIT3	interferon induced protein with tetratricopeptide repeats 3
ENSBTAG00000016662	2.55245	0.000413	0.076938	CPS1	carbamoyl-phosphate synthase 1
ENSBTAG00000000510	-1.195	0.00042	0.076938	ATG101	autophagy related 101
ENSBTAG00000020984	1.28685	0.00041	0.076938	RAPGEF4	rap guanine nucleotide exchange factor 4
ENSBTAG00000034918	2.65752	0.000434	0.077877	IFIT2	interferon induced protein with tetratricopeptide repeats 2
ENSBTAG00000019577	2.01058	0.000443	0.078589	ACKR4	atypical chemokine receptor 4
ENSBTAG00000031750	1.61752	0.000478	0.079042	PLAC8	placenta specific 8
ENSBTAG00000034501	2.42861	0.000458	0.079042	CFI	complement factor I
ENSBTAG00000016275	2.17885	0.00046	0.079042	AMDHD1	amidohydrolase domain containing 1
ENSBTAG00000009215	2.19159	0.000469	0.079042	NR1I3	nuclear receptor subfamily 1 group I member 3
ENSBTAG00000014177	2.5701	0.000467	0.079042	C6	complement C6

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6.5 (contd). Differentially expressed genes in muscle between HF animals divergent for RFI when offered a zero grazed grass diet

	log2FoldChange	pvalue	padj	Symbol	Name
ENSBTAG00000008505	2.58922	0.000502	0.080356	APOB	apolipoprotein B
ENSBTAG00000003120	1.66151	0.000502	0.080356	ZNF385B	zinc finger protein 385B
ENSBTAG00000009876	2.67643	0.000497	0.080356	C4BPA	C4b-binding protein alpha chain
ENSBTAG00000013311	1.20981	0.000508	0.080356		
ENSBTAG00000014529	2.50162	0.000517	0.080602		
ENSBTAG00000016149	2.53404	0.00052	0.080602	C9	complement C9
ENSBTAG00000012801	3.16881	0.000548	0.080602	UPP2	uridine phosphorylase 2
ENSBTAG00000018416	2.07328	0.000542	0.080602	CCDC42	coiled-coil domain containing 42
ENSBTAG00000009392	1.90746	0.000542	0.080602	PLA1A	phospholipase A1 member A
ENSBTAG00000018227	1.64494	0.000553	0.080602	SLC4A7	solute carrier family 4 member 7
ENSBTAG00000019616	2.74197	0.000536	0.080602	APCS	amyloid P component, serum
ENSBTAG00000003326	2.07513	0.000575	0.080977	NAIP	NLR family apoptosis inhibitory protein
ENSBTAG00000015241	1.18753	0.000578	0.080977	EPHX2	epoxide hydrolase 2
ENSBTAG00000013718	2.57036	0.000573	0.080977	GC	GC, vitamin D binding protein
ENSBTAG00000006937	1.43688	0.000579	0.080977	ABCA10	ATP binding cassette subfamily A member 10
ENSBTAG00000032656	2.47776	0.000594	0.081226	CPN2	carboxypeptidase N subunit 2
ENSBTAG00000007049	2.71023	0.000596	0.081226	MBL2	mannose binding lectin 2
ENSBTAG00000010184	2.49596	0.000624	0.082854	G6PC	glucose-6-phosphatase catalytic subunit
ENSBTAG00000001000	3.18782	0.000621	0.082854	CA13	carbonic anhydrase 13
ENSBTAG00000031647	2.48346	0.000627	0.082854	LRG1	leucine rich alpha-2-glycoprotein 1
ENSBTAG00000017797	2.93643	0.00063	0.082854		

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6.5 (contd). Differentially expressed genes in muscle between HF animals divergent for RFI when offered a zero grazed grass diet

	log2FoldChange	pvalue	padj	Symbol	Name
ENSBTAG00000012210	2.55989	0.000657	0.085528	C5	complement C5a anaphylatoxin precursor
ENSBTAG00000046768	3.32401	0.000675	0.087177	IGFBP1	insulin like growth factor binding protein 1
ENSBTAG00000019612	1.03112	0.000728	0.088869	RNASE4	ribonuclease A family member 4
ENSBTAG00000007550	-1.7817	0.000713	0.088869	OLFM2	noelin-2 precursor
ENSBTAG00000009894	2.70979	0.000715	0.088869	SERPINA10	serpin family A member 10
ENSBTAG00000017664	3.08147	0.000703	0.088869	HGF	hepatocyte growth factor
ENSBTAG00000044179	3.75717	0.000726	0.088869	CERS6	ceramide synthase 6
ENSBTAG00000002714	1.24226	0.00073	0.088869	GNAI1	G protein subunit alpha i1
ENSBTAG00000012794	2.43737	0.000752	0.090294	PAH	phenylalanine hydroxylase
ENSBTAG00000005122	2.52116	0.000754	0.090294	KNG1	kininogen 1
ENSBTAG00000046324	2.22609	0.000776	0.092076		
ENSBTAG00000006343	4.07766	0.000796	0.092681	IL1R2	interleukin 1 receptor type 2
ENSBTAG00000001936	1.9932	0.000803	0.092681	PCK1	phosphoenolpyruvate carboxykinase 1
ENSBTAG00000026917	2.4991	0.000802	0.092681		
ENSBTAG00000020829	0.97392	0.000812	0.092689	PTPRK	protein tyrosine phosphatase, receptor type, K
ENSBTAG00000006707	1.02481	0.000857	0.092776	ACSL5	acylCoA synthetase longchain family member 5
ENSBTAG00000037533	1.63809	0.000842	0.092776		
ENSBTAG00000005635	0.66402	0.000852	0.092776	TTC39C	tetratricopeptide repeat domain 39C
ENSBTAG00000046158	2.34285	0.00083	0.092776		Complement factor B
ENSBTAG00000012252	0.9872	0.000843	0.092776	MOCOS	molybdenum cofactor sulfurase
ENSBTAG00000009837	2.62886	0.000843	0.092776	ANKS4B	ankyrin repeat sterile domain containing 4B

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6.5 (contd). Differentially expressed genes in muscle between HF animals divergent for RFI when offered a zero grazed grass diet

	log2FoldChange	pvalue	padj	Symbol	Name
ENSBTAG00000017564	1.50773	0.000872	0.093727	CGN	cingulin
ENSBTAG00000007450	2.02352	0.000885	0.09439	C2	complement C2
ENSBTAG00000010357	1.32563	0.0009	0.094984	ST6GAL1	ST6 beta-galactoside alpha-2,6-sialyltransferase 1
ENSBTAG00000015358	1.90455	0.000903	0.094984	ALDOB	aldolase, fructose-bisphosphate B
ENSBTAG00000038361	2.57371	0.000917	0.095341	SERPINA11	serpin family A member 11
ENSBTAG00000047880	0.95536	0.000926	0.095341		
ENSBTAG00000011062	2.99467	0.000925	0.095341	TDO2	tryptophan 2,3-dioxygenase
ENSBTAG00000010419	1.18871	0.000944	0.096134	CYP2R1	cytochrome P450 family 2 subfamily R member 1
ENSBTAG00000023939	2.25995	0.000952	0.096134		
ENSBTAG00000001247	2.59664	0.000953	0.096134	LECT2	leukocyte cell derived chemotaxin 2
ENSBTAG00000030932	1.56198	0.00096	0.096187	IFI44L	interferon induced protein 44 like
ENSBTAG00000021240	4.01546	0.000987	0.097154	DCSTAMP	dendrocyte expressed seven transmembrane protein
ENSBTAG00000017839	2.29402	0.000983	0.097154	TIAM1	T-cell lymphoma invasion and metastasis 1
ENSBTAG00000018548	0.71774	0.00099	0.097154	INTS7	integrator complex subunit 7
ENSBTAG00000002642	2.95196	0.001049	0.098347	SLC17A2	solute carrier family 17 member 2
ENSBTAG00000008575	1.19424	0.00104	0.098347	CGNL1	cingulin like 1
ENSBTAG00000001598	2.06221	0.001037	0.098347	MAP7	microtubule associated protein 7
ENSBTAG00000014521	0.94275	0.001042	0.098347	UGDH	UDP-glucose 6-dehydrogenase
ENSBTAG00000012630	-1.2319	0.001039	0.098347	PAMR1	peptidase containing associated with muscle regeneration 1
ENSBTAG00000002296	1.13138	0.001034	0.098347	TMEM173	transmembrane protein 173

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6.5 (contd). Differentially expressed genes in muscle between HF animals divergent for RFI when offered a zero grazed grass diet

	log2FoldChange	pvalue	padj	Symbol	Name
ENSBTAG00000014075	2.38821	0.00111	0.099427	ITIH2	inter-alpha-trypsin inhibitor heavy chain 2
ENSBTAG00000037949	2.31958	0.001114	0.099427		
ENSBTAG00000020363	2.87835	0.001108	0.099427	GBA3	cytosolic beta-glucosidase
ENSBTAG00000018843	2.35633	0.001093	0.099427	SERPINA1	serpin family A member 1
ENSBTAG00000020884	1.30942	0.00108	0.099427		
ENSBTAG00000016277	1.0937	0.001096	0.099427		mitochondrial amidoxime reducing component 2 precursor
ENSBTAG00000022395	2.83151	0.001111	0.099427		
ENSBTAG00000020980	0.62911	0.001108	0.099427	CAT	catalase
ENSBTAG00000024115	-1.5095	0.001146	0.09998	FOXO6	forkhead box O6
ENSBTAG00000004281	0.7625	0.001142	0.09998	ACSS1	acetyl-coenzyme A synthetase 2-like, mitochondrial
ENSBTAG00000017056	2.32046	0.001146	0.09998	PKLR	pyruvate kinase, liver and RBC

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6.6. Differentially expressed genes in muscle between CH animals divergent for RFI when offered a high concentrate diet in their finishing phase.

	log2FC	Pvalue	padj	Symbol	Name
ENSBTAG00000016121	-18.04119739	2.01E-10	4.04E-06	KRT75	keratin 75
ENSBTAG00000043250	5.680925293	2.98E-07	0.002995	7SK	7SK RNA
ENSBTAG00000044427	6.491227371	7.79E-07	0.005221	RNaseP_nuc	Nuclear RNase P
ENSBTAG00000043086	5.550003532	1.35E-05	0.068146	Y_RNA	Y RNA

Log2FC = log 2 fold change; padj = adjusted p-value

Table 6.7. Functional analysis using both the up- and down- regulated genes from DAVID in high and low RFI HF steers offered a zero grazed grass diet.

Annotation Cluster 1	**Enrichment Score: 9.54**	**P_Value**	**Benjamini**
	Secreted	4.00E-15	6.90E-13
	Signal	8.70E-11	5.00E-09
	disulfide bond	1.60E-10	5.50E-08
	Glycoprotein	3.70E-10	1.30E-08
	signal peptide	5.80E-09	9.90E-07
	Disulfide bond	2.20E-08	5.60E-07
	glycosylation site:N-linked (GlcNAc...)	6.00E-08	5.10E-06
Annotation Cluster 2	**Enrichment Score: 7.17**	**P_Value**	**Benjamini**
	Innate immunity	6.10E-11	5.30E-09
	Immunity	1.80E-08	5.20E-07
	innate immune response	2.80E-04	2.40E-02
Annotation Cluster 3	**Enrichment Score: 5.43**	**P_Value**	**Benjamini**
	Blood coagulation	2.70E-10	1.20E-08
	Hemostasis	2.70E-10	1.20E-08
	Fibrinolysis	2.70E-04	2.70E-02
	receptor binding	3.00E-03	2.00E-01
	Calcium	1.20E-02	8.60E-02

Table 6.7 (contd). Functional analysis using both the up- and down- regulated genes from DAVID in high and low RFI HF steers offered a zero grazed grass diet.

Annotation Cluster 4	**Enrichment Score: 5.04**	**P_Value**	**Benjamini**
	CCP	1.80E-08	1.90E-06
	Sushi/SCR/CCP	2.20E-08	9.40E-06
	domain:Sushi 1	5.30E-08	6.00E-06
	domain:Sushi 2	7.50E-08	5.10E-06
	Sushi	2.30E-06	4.40E-05
	domain:Sushi 3	8.30E-06	4.70E-04
	domain:Sushi 7	4.10E-04	1.70E-02
	domain:Sushi 8	4.10E-04	1.70E-02
	domain:Sushi 6	8.10E-04	2.70E-02
	domain:Sushi 5	2.00E-03	6.00E-02
	domain:Sushi 4	4.70E-03	1.20E-01
Annotation Cluster 5	**Enrichment Score: 3.54**	**P_Value**	**Benjamini**
	Complement pathway	1.40E-07	3.00E-06
	complement activation, classical pathway	2.60E-07	1.40E-04
	Pertussis	9.50E-03	1.90E-01
	Prion diseases	4.60E-02	4.50E-01

Table 6.7 (contd). Functional analysis using both the up- and down- regulated genes from DAVID in high and low RFI HF steers offered a zero grazed grass diet.

Annotation Cluster 6	**Enrichment Score: 3.16**	**P_Value**	**Benjamini**
	Serpin family	4.40E-05	9.30E-03
	Serpin domain	4.40E-05	9.30E-03
	SERPIN	5.00E-05	2.60E-03
	Protease inhibitor	1.80E-04	2.50E-03
	serine-type endopeptidase inhibitor activity	2.10E-04	4.60E-02
	negative regulation of endopeptidase activity	2.20E-04	2.80E-02
	Serine protease inhibitor	2.20E-04	2.70E-03
	Protease inhibitor I4, serpin, conserved site	3.90E-04	3.30E-02
	chromaffin granule	3.40E-03	5.90E-02
	Cytoplasmic vesicle	2.70E-01	7.80E-01
	cytoplasmic vesicle	3.00E-01	9.60E-01
Annotation Cluster 7	**Enrichment Score: 2.89**	**P_Value**	**Benjamini**
	Staphylococcus aureus infection	1.90E-07	1.40E-05
	domain:Peptidase S1	9.50E-05	4.60E-03
	Peptidase S1A, chymotrypsin-type	3.90E-04	4.00E-02
	Peptidase S1	6.40E-04	4.40E-02
	Tryp_SPc	6.60E-04	1.70E-02
	Trypsin-like cysteine/serine peptidase domain	8.70E-04	4.50E-02
	serine-type endopeptidase activity	4.40E-03	1.50E-01
	active site:Charge relay system	8.70E-03	1.90E-01
	Peptidase S1, trypsin family, active site	1.10E-02	3.10E-01
	Serine protease	1.40E-02	9.40E-02
	Proteolysis	2.40E-02	5.90E-01

Table 6.7 (contd). Functional analysis using both the up- and down- regulated genes from DAVID in high and low RFI HF steers offered a zero grazed grass diet.			
Annotation Cluster 8	**Enrichment Score: 2.37**	**P_Value**	**Benjamini**
	Monooxygenase	4.60E-04	5.30E-03
	Secondary metabolites biosynthesis, transport, and catabolism	5.30E-04	5.30E-03
	Iron	5.80E-04	6.20E-03
	heme binding	1.30E-03	1.40E-01
	Heme	2.40E-03	2.10E-02
	Oxidoreductase	2.80E-03	2.40E-02
	iron ion binding	3.10E-03	1.60E-01
	Cytochrome P450, E-class, group I	8.10E-03	3.20E-01
	Cytochrome P450, conserved site	1.10E-02	3.00E-01
	Cytochrome P450	1.60E-02	3.70E-01
	steroid hydroxylase activity	1.70E-02	3.50E-01
	monooxygenase activity	4.10E-02	5.70E-01

Table 6.7 (contd). Functional analysis using both the up- and down- regulated genes from DAVID in high and low RFI HF steers offered a zero grazed grass diet.			
Annotation Cluster 9	**Enrichment Score: 2.3**	**P_Value**	**Benjamini**
	Fibrinogen, alpha/beta/gamma chain, coiled coil domain	2.50E-04	3.40E-02
	SM01212	2.70E-04	9.20E-03
	platelet activation	1.40E-03	8.60E-02
	fibrinogen complex	1.60E-03	3.90E-02
	receptor binding	3.00E-03	2.00E-01
	protein polymerization	3.70E-03	1.90E-01
	protein binding, bridging	1.10E-02	3.00E-01
	Platelet activation	1.60E-01	7.30E-01
	signal transduction	7.30E-01	1.00E+00
Annotation Cluster 10	**Enrichment Score: 1.81**	**P_Value**	**Benjamini**
	Glycolysis / Gluconeogenesis	4.70E-03	1.60E-01
	Biosynthesis of antibiotics	6.70E-03	1.90E-01
	Carbon metabolism	3.00E-02	4.00E-01
	Pyruvate metabolism	6.30E-02	4.90E-01
Annotation Cluster 11	**Enrichment Score: 1.81**	**P_Value**	**Benjamini**
	domain:Fibrinogen C-terminal	8.40E-03	2.00E-01
	Fibrinogen, conserved site	9.20E-03	3.20E-01
	Fibrinogen, alpha/beta/gamma chain, C-terminal globular, subdomain 2	1.60E-02	3.80E-01
	FBG	1.70E-02	2.90E-01
	Fibrinogen, alpha/beta/gamma chain, C-terminal globular, subdomain 1	2.50E-02	4.50E-01

Table 6. 7 (contd). Functional analysis using both the up- and down- regulated genes from DAVID in high and low RFI HF steers offered a zero grazed grass diet.			
Annotation Cluster 12	**Enrichment Score: 1.24**	**P_Value**	**Benjamini**
	Low-density lipoprotein (LDL) receptor class A, conserved site	4.50E-02	6.20E-01
	LDLa	6.30E-02	6.70E-01
	Low-density lipoprotein (LDL) receptor class A repeat	6.60E-02	7.50E-01
Annotation Cluster 13	**Enrichment Score: 0.83**	**P_Value**	**Benjamini**
	sequence variant	4.50E-02	6.30E-01
	Alternative splicing	2.10E-01	7.00E-01
	splice variant	3.50E-01	1.00E+00
Annotation Cluster 14	**Enrichment Score: 0.75**	**P_Value**	**Benjamini**
	Insulin signaling pathway	5.90E-02	5.10E-01
	Glucagon signaling pathway	8.30E-02	5.60E-01
	AMPK signaling pathway	1.40E-01	7.00E-01
	Insulin resistance	3.40E-01	9.20E-01
	PI3K-Akt signaling pathway	7.30E-01	1.00E+00
Annotation Cluster 15	**Enrichment Score: 0.65**	**P_Value**	**Benjamini**
	EGF-like domain	3.20E-02	2.00E-01
	EGF-like, conserved site	2.10E-01	9.80E-01
	EGF-like calcium-binding	2.70E-01	9.90E-01
	EGF_CA	2.80E-01	9.90E-01
	EGF	4.40E-01	1.00E+00

Table 6.7 (contd). Functional analysis using both the up- and down- regulated genes from DAVID in high and low RFI HF steers offered a zero grazed grass diet.

Annotation Cluster 16	**Enrichment Score: 0.57**	**P_Value**	**Benjamini**
	Chemokine receptor family	1.00E-02	3.30E-01
	chemotaxis	1.10E-02	4.30E-01
	Receptor	9.90E-01	1.00E+00
	Transducer	1.00E+00	1.00E+00
	G-protein coupled receptor	1.00E+00	1.00E+00
	G protein-coupled receptor, rhodopsin-like	1.00E+00	1.00E+00
	GPCR, rhodopsin-like, 7TM	1.00E+00	1.00E+00
Annotation Cluster 17	**Enrichment Score: 0.28**	**P_Value**	**Benjamini**
	GTP-binding	3.40E-01	8.30E-01
	nucleotide phosphate-binding region:GTP	6.10E-01	1.00E+00
	GTP binding	6.80E-01	1.00E+00
Annotation Cluster 18	**Enrichment Score: 0.27**	**P_Value**	**Benjamini**
	Nucleotide-binding	2.90E-01	8.00E-01
	ATP-binding	6.20E-01	9.70E-01

Table 6.7 (contd). Functional analysis using both the up- and down- regulated genes from DAVID in high and low RFI HF steers offered a zero grazed grass diet.

Annotation Cluster 19	**Enrichment Score: 0.26**	**P_Value**	**Benjamini**
	Immunoglobulin domain	3.90E-01	8.70E-01
	Immunoglobulin-like domain	5.70E-01	1.00E+00
	Immunoglobulin subtype	5.90E-01	1.00E+00
	IG	6.10E-01	1.00E+00
	Immunoglobulin-like fold	6.50E-01	1.00E+00
Annotation Cluster 20	**Enrichment Score: 0.08**	**P_Value**	**Benjamini**
	topological domain:Extracellular	6.20E-01	1.00E+00
	topological domain:Cytoplasmic	8.10E-01	1.00E+00
	Cell membrane	8.30E-01	1.00E+00
	plasma membrane	9.80E-01	1.00E+00
Annotation Cluster 21	**Enrichment Score: 0.07**	**P_Value**	**Benjamini**
	Membrane	7.70E-01	9.90E-01
	Transmembrane	8.70E-01	1.00E+00
	integral component of membrane	8.80E-01	1.00E+00
	Transmembrane helix	9.00E-01	1.00E+00
Annotation Cluster 22	**Enrichment Score: 0.01**	**P_Value**	**Benjamini**
	Transcription regulation	9.80E-01	1.00E+00
	transcription, DNA-templated	9.80E-01	1.00E+00
	Transcription		

Table 6.8. Top canonical pathways related to RFI in muscle tissue from HF animals consuming zero grazed grass as identified by IPA.

Canonical Pathway	*P*-value
IL-6 Signaling	7.33E-04
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	8.39E-04
NF-B Signaling	1.46E-03
Serotonin and Melatonin Biosynthesis	1.96E-03
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	2.40E-03

6.3 Discussion

Understanding the metabolic and physiological mechanisms underlying variation in feed efficiency in cattle is vital for the effective planning of breeding strategies to select the most feed efficient cattle [180]. Research examining the biological mechanisms governing RFI, and other measures of feed efficiency; have mainly focused on young cattle offered high-concentrate diets. In practice, however, most commercial beef production systems worldwide are predominantly forage based [351] exploiting compensatory growth during backgrounding, with animals subsequently finished on a concentrate based diet. RFI is a complex trait that is affected by both genetic and environmental factors with breed and diet having a major influence [26, 346]. However, it is not clear from the literature how breed and diet impact on the regulation of RFI in cattle. Furthermore, if predictive biomarkers for RFI are to be utilised they must be robust across breed and diet. This study aimed to determine the key regulatory genes in pathways and networks differentially expressed in cattle divergent for RFI within two contrasting breeds and across diverse diets with a view to elucidating the key biological mechanisms controlling the expression of RFI in muscle tissue. The key findings from this research are: i) that there is no consistent biological mechanism governing variation for RFI in muscle tissue across CH and HF cattle breeds sequentially offered different diets; ii) that within each diet there appears to be different biological mechanisms governing RFI in muscle, demonstrating the possibility of genotype x environment at least at the level of muscle metabolism and iii) that an altered immune response may be contributing to variation in RFI in HF animals while consuming fresh forage.

It is important to note that following DEG analysis and a subsequent correction for multiple tests, only 2 out of 6 high versus low RFI comparisons resulted in significant FDR values. This is similar to previous reports in relation to feed efficiency [317, 318, 352] and certainly data from our own group has returned similar findings (Chapter 5). While multiple testing correction is worthwhile and provides confidence in the observed results, it is also possible that reducing

the number of the proportion of tests mistakenly declared significant, may come with the cost of increasing false negatives and subsequently masking true results [353]. Nonetheless, we have chosen to discuss results only passing multiple testing corrections.

Currently, research on the transcriptional control of feed efficiency in cattle, is focused on the identification of genetic variants of biological significance to the trait, within breed. Different breeds of cattle have varying capacities to transform nutrients into energy and subsequently there is large variation in feed efficiency [12] between breeds. It is widely accepted, for example, that HF have a higher intake capacity and lower carcass development characteristics than CH cattle. It follows that any biological mechanisms that are consistently observed to be involved with RFI across both of these breeds must be extremely robust therefore having the potential to be explored as biomarkers for this trait across breeds. Furthermore, RFI studies conducted using the HF breed are predominantly concerned with lactating cows and heifers [354-356] when in fact, HF males are used exclusively for beef production highlighting the necessity for this research. In the current study we failed to observe any genes or biological functions to be consistently associated with RFI across the two breeds. While this result was unexpected, it is important. As mentioned in the materials and methods section, this study was conducted as part of a larger study conducted by Coyle et al. (2017) [62], and as expected the two breeds were quite divergent in performance traits and FE. The two breeds did not differ in ADG over any of the three dietary periods studied; however, the DMI of the CH cattle were significantly lower for each feeding phase. This is consistent with many other studies in which CH have been found to be far more efficient in utilising their feed than HF [357]. We hypothesise that the absence of a consistent biological mechanism governing feed efficiency between the two breeds is due to their different physiology, and, this finding provides evidence that energetic efficiency is regulated in different ways across cattle breeds. While the differing capacities of breeds to convert nutrients to energy is not wholly understood [358], this finding indicates that feed efficiency and in particular RFI may need to be selected for within breed and

our data may be a starting point for revealing the transcriptional control of nutrient utilisation between breeds.

It is well established that intake regulation varies depending on diet type. For example, an animal can fill up on forage before meeting basic nutritional requirements and indeed the same animal will meet those requirements more readily on a high concentrate regime [346]. It therefore follows that diet could have a significant impact on gene expression associated with intake variation and feed efficiency and it has been well documented that offering cattle high concentrate compared with high forage diets can alter expression of genes associated with many biological processes [359, 360]. In the current study we examined if there were any genes or biological mechanisms consistently implicated with RFI, regardless of diet. Interestingly, in the current study, there were no genes or biological pathways that were consistently differentially regulated between the animals divergent in feed efficiency across diets. This finding, supporting the deductions of Coyle et al. 2017) [62] where diet was only relatively repeatable, with approximately half of the animals maintaining their RFI classification between phases. Given that, cattle are generally reared on high forage diets and finished on high concentrate diets, identifying specific animals that maintain their RFI status across these diets may be the key to uncovering the biology behind this multi-faceted trait. To the author's knowledge, these deductions provide evidence for the first time that, developing a biomarker for RFI, that would robustly predict a feed efficient animal across breed and regardless of diet, may be difficult.

Regulation of intake is influenced heavily by the stage of physiological maturity in cattle [361] and an animal's metabolism can shift over the course of its lifetime. This study had an added benefit, in that, data from contemporary CH and HR cattle at different production stages cosuming the same diet was available (Phase 1 and 3). We observed no genes or mechanisms that were commonly altered in response to RFI between young animals offered a high concentrate diet and subsequently offered high concentrates in their finishing stage. This

finding indicates that RFI values obtained at different stages of maturity may not be comparable.

Despite the lack of consistency across diet and breed, we did observe some differential gene expression between high and low RFI animals within breed and diet comparisons. HF steers divergent for RFI, and offered zero grazed grass had differential expression of 170 genes (Table 6.8) When these DEG were analysed using IPA, the three top canonical pathways were related to immune function indicating that the less efficient animals have an increased immune response when managed under such a dietary regimen. This finding is further supported by the work of Paradis et al. (2015) [301] who concluded that more efficient cattle have a lower immune response than their less efficient counterparts. Similarly, Alexandre et al. (2015) observed an enrichment of the inflammatory response in the liver of low feed efficient cattle [362]. These results are not surprising given that the immune response is metabolically very costly [363] and could potentially have a negative effect on an animal's feed efficiency. In addition, the animals in this dietary phase could be considered to be in a state of stress as they had changed from a high concentrate diet to zero grazed grass which is a less energy dense and nutritious diet. Perhaps the high RFI animals had a higher feed intake in order to support their elevated immune response. Furthermore, a study in swine suggested that pigs that have a low RFI phenotype might have an energy saving mechanism, by which the immune response is dampened in the intestine [364]. IPA also identified one network associated with RFI that was related to organismal injury and abnormalities, gastrointestinal disease and inflammatory disease. Interestingly, none of the molecules involved in this network have previously been related to feed efficiency in cattle. We hypothesise that this upregulation in disease networks and immunological functions in the less efficient animals may be attributed to their lack of capacity to deal with the change to zero grazed grass when compared to their more efficient counterparts.

A number of the most extreme DEG in the current study have previously been associated with RFI, namely, *HSPA6 (heat shock 70kDa protein 6)* [301], *SLC16A1 (monocarboxylate transporter 1)* [364] and *DDPIV (dipeptodyl peptidase 4*) [365]. The protein encoded by *HSPA6* is a member of the *HSP70* family, which plays an important role in quality control of protein folding and generally protecting the proteome from stress. Members of the heat shock protein family have previously been associated with feed efficiency [181, 287, 297, 301, 316] and in addition data from our own group (Chapter 5) have observed a relationship between RFI and *HSPA5.* However, in contrast to the literature mentioned above, in the current study we observed an upregulating of the *HSPA6* gene in the muscle of the high RFI, less efficient HF animals. This could indicate that the less efficient animals are producing more of this protein to combat excess stress on the proteome. In cattle, *SLC16A1* plays an important role in the cellular responses to changes in diet by modulating the cellular levels of lactate and pyruvate. *SLC16A1* has been previously associated with feed efficiency [364]. In addition, the protein encoded by this gene is involved in modulating insulin secretion with simultaneous effects on plasma insulin levels and blood glucose homeostasis [366]. While plasma glucose levels were not affected by RFI in the animals used in the current study [62], *SLC16A1* was found to be upregulated in the high RFI HF animals. The rapid transport of monocarboxylates such as pyruvate, lactate, and the ketone bodies (acetoacetate and β-hydroxybutyrate) across the plasma membrane of cells is essential for carbohydrate, fat, and amino acid metabolism and is facilitated by proton-linked monocarboxylate transporters such as *SLC16A1* [366]. An upregulation in the activity of this protein is indicative of cellular stress [366] and suggests that the inefficient animals in the current study may not be as cellularly stable as their efficient counterparts. Finally, the less efficient animals had an upregulation of the *DDPIV* gene. The enzyme encoded by this gene is rather indiscriminate and has a variety of substrates [367]. In addition, when a gene knockout study of this enzyme is carried out in mice, the mice show protection against obesity and insulin resistance [368]. This would suggest that the less efficient

animals in the current study are more prone to metabolic diseases such as obesity and insulin resistance.

We observed 4 genes to be differentially to be upregulated in the high RFI CH steers offered a high concentrate diet during their finishing phase which were *KRT75, 7SK RNA, RNaseP nuc* and *Y RNA.* While the significance of *KRT75* in relation to FE is unclear, the three latter genes are all involved in gene expression regulation. Small non-coding *RNAs* such as *7SK RNA* and *Y RNA* are increasingly recognized to play crucial roles in both, pre- and post-transcriptional regulation of gene expression [369]. The upregulation in these genes in the present study may be an indication of differential transcriptional regulation of key proteins between animals divergent for RFI. Similarly, *Nuclear RNase P* is a ribonuclease involved in RNA processing by cleaving RNA. These observations warrant a further, more detailed examination, in order to understand the regulation of differential gene expression in cattle divergent for phenotypic feed efficiency.

In conclusion evidence is provided that effects of RFI on gene expression in muscle of beef cattle are not consistent across breed type or dietary phase. These novel observations challenge the practicality of selecting and breeding for low RFI animals and expecting these to maintain their efficiency across different diets and stages of development. Perhaps, future research should be directed at understanding the biological mechanisms that dictate the advantage of particular genotypes within the context of different dietary regimen and and/or stage of maturity.

Chapter 7

CHAPTER 7. OVERALL SUMMARY, DISCUSSION AND CONCLUSIONS

7.1 Introduction

The global human population is increasing rapidly and the demand for food is predicted to increase by approximately 70% by 2050 [370]. Such requirements can only be facilitated by an increase in production efficiency of both animal and plant derived products [298]. However, within this resource-constrained setting, the rising world demand for food, including meat, must be met while taking cognizance of the environmental footprint of such endeavours.

While feed efficient animals are of great interest from an environmental standpoint, their economic value is of particular importance, given the inherent poor profitability within the beef sector [371]. As stated elsewhere in this thesis, provision of feed represents the single largest variable cost in beef production systems [372], however, unlike the pig and poultry industries where significant advances have been made in feed efficiency, this has not transpired within beef production systems [6]. Improving cattle feed efficiency, by way of selecting for low RFI animals, is acknowledged globally as an attractive approach towards meeting economic and environmental sustainability goals.

While a trait with obvious merit, measuring RFI in cattle is logistically problematic and extremely costly. Thus, there has been considerable worldwide interest in development of predictive biomarkers for the trait which could be harnessed to accurately identify, select and subsequently proliferate the genetics of energetically efficient animals through national breeding programmes. In order to achieve this goal, however, the molecular mechanisms underpinning this complex and multifaceted trait must be fully understood. For example, Herd and Arthur (2009) estimated that multiple biological processes are likely to contribute to variation in RFI in beef cattle [48]. These include processes such as body tissue composition, feeding patterns, activity, digestibility, heat increment of fermentation, tissue metabolism and protein turnover as well stress and immune response. However, this comprehensive list is not

exhaustive and the authors estimated that all of the aforementioned processes are cumulatively likely to explain only 70% of the variation in RFI [48]. Despite being the topic of much recent study, the molecular mechanisms governing RFI remain largely unknown [44]. In addition, most of the studies undertaken to-date have typically used only one breed and or gender of cattle typically offered a high concentrate, energy dense diet. While the high concentrate, finishing phase is considered the single most expensive phase of beef production, and is important from and economic standpoint, forage based diets are the mainstay of beef cattle production systems worldwide [44]. The literature pertaining to the within animal stability of RFI classification across different diets and stages of physiology is scant despite the fundamental importance of such factors in determining the lifetime biological efficiency of an animal.

The aims of this thesis were to (i) increase our understanding of the underlying biology governing feed efficiency, as measured by RFI in a number of metabolically important tissues, (ii) to examine the effect of RFI status, gender and diet as well as their interactions on targeted and global transcriptomic expression across three metabolically important tissues (iii) to complement the aforementioned research with functional measures of mitochondrial biochemistry.

To meet these objectives, the experimental work in this thesis, as outlined in Chapter 2, was multidisciplinary in nature and involved measurement of feed intake and productivity-related traits such as live weight, growth, body composition and blood metabolites. In addition, mitochondrial functional assays, real time quantitative polymerase chain reaction, and global transcriptomics in the form of RNA sequencing were used to examine the biochemical and molecular aspects of interest.

It is estimated that a large proportion of variation in feed efficiency can be accounted for by differences in biochemical mechanisms governing energy production in metabolically

significant tissues [48]. Furthermore, mitochondria are responsible for 90% of energy production in cells in mammals [229]. While some studies have attempted to examine the relationship between phenotypic energetic status in cattle and mitochondrial biochemistry/functionality in target tissues, the findings of these studies are equivocal. Therefore, in Chapter 3, a study to examine the effect of RFI classification on mitochondrial abundance and functionality in muscle and liver from beef cattle was conducted.

Many studies have reported positive associations between RFI and carcass adiposity in cattle. Indeed Karisa et al. (2013) analysed major gene networks associated with RFI and observed that the trait was associated with lipid and steroid metabolism, in particular lipogenesis and cholesterol metabolism [283]. In addition, Weber et al. (2016) concluded that there was a deactivation of the regulatory networks controlling fatty acid metabolism in visceral AT of low RFI animals [182]. The biological mechanisms underlying this putative relationship are not fully understood. Thus, in Chapter 4 the effect of RFI on the expression of key candidate genes related to lipid metabolism in adipose tissue was examined.

Global transcriptomic analysis is a powerful approach to provide comprehensive insight into the molecular mechanisms underpinning complex traits such as RFI in metabolically important tissues and has been employed by a number of recent studies investigating the mechanisms contributing to the molecular control of feed efficiency [34, 182, 317-319, 329, 336]. Despite this, the results to-date from these studies in beef cattle are vague and inconsistent. This may be a result of differences between studies in the breed type, gender, nutritional management regimen and tissues examined. Therefore, in order to more equitably examine the effect of RFI status on global gene expression in two key metabolically important tissues, RNA-Seq technology was harnessed to study the transcriptome of both muscle and liver tissues from purebred bulls and heifers divergent within gender for RFI and reared together, from birth as a contemporary group (Chapter 5).

RFI is a complex trait affected by both genetic and environmental factors [26, 346]. The literature pertaining to the impact of breed and diet and, in particular, their interaction, on the transcriptional regulation of RFI in metabolically important tissues in beef cattle is scarce. Furthermore, if predictive biomarkers for RFI are to be utilized they must be robust across both breed and diet. Thus, the aim of the study described in Chapter 6, was to determine the key regulatory genes and pathways contributing to variation in RFI across contrasting breeds and diets.

7.2 A word on experimental design

The nature of RFI is complex and multifaceted. RFI value results from determining the difference between actual and expected DMI for a given bodyweight and rate of weight gain. The expected DMI for a particular animal is calculated from the group regression parameters derived from its contemporaries based on their measured performance. This calculation is unique for a particular contemporary group and is a complicating aspect in determining the final result, limiting the equitable comparison of RFI coefficients between groups of animals [299]. The animals studied in Chapters 3, 4 and 5 were purebred SM heifers and bulls offered an *ad libitum* diet based on energy dense concentrate feed. Fat deposition can be viewed as an additional energy sink in livestock and there is evidence in the literature that incorporating an adjustnment for backfat accretion within the selection parameters for improved RFI can mitigate against the reported antagonistic relationship between energetic efficiency and carcass adiposity [34, 373]. Therefore, the RFI calculation pertaining to the cohort of animals employed in the studies conducted for Chapters 3-5 included an adjustment for backfat thickness. Chapter 6 of this thesis examined RFI in CH and HF steers managed across three distinct dietary phases and in contrast to the other chapters, backfat thickness was not adjusted for in the RFI calculation. As previously mentioned in Chapter 2, this study was conducted as part of a larger experiment designed to measure the repeatability of RFI across diet [62]. Interestingly, a concluding remark of the authors was that fat may need to be included in the base model used

to predict feed intake in order to eliminate possible antagonistic effects. While one may argue that the RFI calculation methodolgy utilized in this thesis should have been consistent, the view could also be taken that the approach taken represents the different methodologies reported in the published literature.

Furthermore, it is well established that there is large variation in feed efficiency between breeds and this is further exasperated when different diets are fed [374]. The use of SM, CH and HF breeds consuming a variety of diets in this thesis, attempted to capture information on the diverse biological mechanisms contributing to divergence in RFI.

Finally, this thesis examined the effects of divergent ranking of animals for RFI on multiple metabolically active tissues, muscle, liver and adipose and also on the blood plasma profile. While there is evidence of an association between the energetic processes in these tissues and RFI [229], the precise mechanisms in beef cattle are still largely unknown.

7.3 Mitochondria

There is evidence that feed efficient mice have enhanced mitochondrial biogenesis, however, there is a void in the current knowledge base on this concept for beef cattle [229]. The results of Chapter 3 indicate that mitochondrial abundance, as quantified by way of citrate synthase assay, in muscle and liver tissue does not vary with RFI status in beef cattle. Many cross species studies have both hypothesized and observed a link between the functional capacity of mitochondria and RFI. Bottje and Carstens (2012) have demonstrated that inefficient animals may have increased proton leakage from their inner mitochondrial membrane resulting in a decrease in cellular energy production [375]. Moreover, it has been determined that cellular oxidative phosphorylation is more tightly regulated in feed efficient animals [376] resulting in less ROS production [375]. The efficiency of oxidative phosphorylation was examined in Chapter 3 by measuring the activity of the enzymatic complexes that comprise the electron transport chain. There were no differences in complex activity in muscle tissue between RFI

phenotypes. Similarly, complex activity was not altered in the liver aside from complex IV, which was higher in the less efficient animals in agreement with the observations of Kolath et al. (2006a) [220]. When examining the electron transport chain and oxidative phosphorylation as a whole, the results reported in Chapter 3 suggest that there is no effect of RFI status on the function of the electron transport chain in beef cattle, consistent with the findings of [221, 377]. Many recent publications have examined differential expression of genes related to oxidative phosphorylation in order to determine the contribution of this process to variation in RFI. Kelly et al. (2011b) and Fonseca et al. (2015) reported upregulation of the uncoupling proteins *UCP3* in muscle [247], and *UCP2* in liver [378] from less efficient animals, respectively. *PPARGC1α* which is a major player in controlling energy homeostasis, through mitochondria, was found to be upregulated in muscle from feed efficient animals [247, 297]. Moreover, *COX2* gene expression was elevated in muscle tissue from feed efficient heifers [247] further supporting the hypothesis of enhanced efficiency of oxidative phosphorylation in beef cattle. The data reported in Chapter 5 of this thesis are consistent with this hypothesis. When global gene expression was analyzed in muscle tissue from heifers divergent in RFI, it was observed that all 5 DEG encoded proteins were involved in the electron transport chain. In addition, consistent with the results of multiple studies [225, 238, 247, 322] these 5 DEGs (*COX1 (cytochrome c oxidase subunit 1), ND5 (NADH-dehydrogenase 5), ND6 (NADH-dehydrogenase 6), CYTB (cytochrome b), COX3 (cytochrome c oxidase subunit 3)* were upregulated in the more efficient animals.

Interestingly, the results observed in Chapters 3 and 5 pertained to the same animal model. While an increase in activity of the electron transport chain in the more efficient animals was apparent in the transcriptome, these increases were not reflected at the functional level, indicating that the magnitude of the change observed at the transcriptional level is insufficient to influence functionality or that perhaps cellular transcripts within the efficient animals

undergo post transcriptional modifications. This subject warrants further investigation, in the form of comprehensive proteomic analysis.

7.4 Lipogenesis

Adipose tissue is involved heavily in appetite regulation and subsequently makes a significant contribution to overall body homeostasis. Furthermore, differential utilization of dietary and tissue derived lipids and carbohydrates has been suggested to contribute to variation in feed efficiency in beef cattle [91]. Consistent with this premise, it is not unreasonable to suggest that differences may exist between animals of varying feed efficiency potential, in biochemical processes governing nutrient partitioning and fat deposition [48]. The biochemical and molecular background affecting genetic variability for nutrient partitioning in cattle remain largely unknown [379]. Overexpression of *GLUT4* in adipocytes of transgenic mice has been observed to result in increased glucose metabolism in all major pathways, with differential regulation of the pathways involved in lipogenesis [380]. The main finding of Chapter 4 was that the divergence for RFI across gender affects the gene expression of *GLUT4,* the rate limiting step of glucose uptake from the bloodstream by AT [380]. This finding indicates that the decreased expression of *GLUT4* in the high RFI animals represents less efficient glucose homeostasis and the potential for increased insulin resistance in these animals. Consistent with these results are the observations of Karisa et al. (2013) who predicted that biological processes associated with variation in RFI could begin with glucose uptake into the cell through *GLUT4* [283]. Similarly, Kelly et al. (2011) reported greater levels of circulating plasma insulin in low RFI animals [247] and as insulin stimulates the activity of *GLUT4* this supports the current studies' finding of the increased *GLUT 4* in the low RFI phenotype. In addition, in the study of Keogh et al. (2015), it was suggested that improved insulin sensitivity of feed efficient cattle may be contributing to reduced lipogenesis and adipose deposition [381]. While the observations of Fitzimons et al. (2014), which were deduced on the same herd of animals as in Chapter 4, found no relationship between insulin response, sensitivity and gene expression in

muscle and RFI, *SREBPc1* was differentially expressed in response to RFI. The expression of *SREBPc1*, which is involved in lipogenesis, was examined in AT in Chapter 4, however no relationship with RFI was identified.

The findings of Chapter 4 also indicate that, within gender, different mechanisms in AT may be contributing to variation in RFI. In bulls, differential expression of 3 key genes involved in the lipogenesis pathway in adipose tissue, were associated with divergence for RFI. Expression of *ACLY* was elevated in response to the high RFI phenotype. This observation suggests that the less efficient bulls are directing molecular mechanisms related to substrate partitioning towards fat deposition which is consistent with the hypothesis of multiple studies [44, 305]. In contrast, both *HMG-CoAS* and *ACAT1* were upregulated in the more efficient heifers. Increase of *HMG-CoAS*, which is involved in the initiation of ketogenesis, in times of carbohydrate deprivation [382] may be accounted for simply by the lower feed intake of these animals. An important role of *ACAT1* is to protect the cell against toxic buildup of unesterified cholesterol in the cell membrane [383]. The findings of Chapter 4, in relation to this enzyme indicate that the more efficient heifers have an increased mechanism to alleviate the buildup of unesterified cholesterol.

7.5 Transcriptomics

The main limitation to widespread adoption of feed efficiency in cattle breeding programmes are the large cost and technical difficulty associated with measuring the trait [384]. As a consequence, the discovery of reliable biomarkers for RFI, which would reduce the cost of identifying and breeding efficient animals, has become of paramount importance [329]. The use of gene transcript expression and pathway analysis, as conducted in Chapters 5 and 6, in order to understand the biological mechanisms underlying RFI at tissue level, is a useful and informative step in the development of DNA based biomarkers that could be incorporated into a genomic selection based breeding strategy.

Transcriptomic analyses conducted as part of the studies outlined in Chapters 5 and 6 were applied to three distinct breeds, SM, CH and HF; different genders, heifers, bulls and steers; and diets of varying chemical composition and nutritive value (concentrate v forage). In both Chapters 5 and 6 many DEG were identified between high and low RFI animals. However, in certain comparisons the DEG failed to pass the strict statistical analysis criteria employed. We hypothesised that due to the fact that RFI is a ranking system within a given population, these results could be due to the relatively low within contemporary group variation observed in this study. These results are common for RFI studies due to the complex nature of the trait and many studies have published DEG lists that did not obtain an adequate FDR value (< 0.1) in relation to feed efficiency in cattle [317-319]. In Chapters 5 and 6 the decision to only include DEG that passed multiple correction analysis (FDR <0.1) was to ensure that any DEG associated with RFI were selected with a high degree of confidence and thus robust in nature.

In Chapter 5, transcriptomic and pathway analysis via DAVID and IPA software approaches revealed, that efficient heifers had an enhanced capacity for mitochondrial function in muscle tissue, consistent with the findings of [181, 225, 238, 247, 322]. The same analyses determined that oxidative response, protein mediation and cell signaling in the liver are likely to be processes that are influencing variation in feed efficiency in bull liver tissue which are consistent with the observations of [287, 296, 301, 316, 319]. The findings of Chapter 6 in conjunction with that of Paradis et al. (2015) and Alexandre et al. (2015) [301, 362], highlight a relationship between RFI and immune function. Efficient HF steers had evidence for a lower immune status than their less efficient counterparts though only when offered zero grazed grass. Given that the immune system is metabolically costly, and animals consuming zero grazed grass after a period of high concentrates could be considered to be in a state of nutritional stress, it is possible that the more efficient animals employ a better coping mechanism by damping their immune response. Consistent with this hypothesis are mulitple studies that have proposed that

more efficient pigs [364] and birds [385] are more effective at controlling expression of immune response genes.

In Chapter 6, evidence is provided that effects of RFI on gene expression in muscle of beef cattle are not consistent across breed type or dietary phase. Furthermore, when considered with the findings of Chapter 5, it is clear that there is no consistent biological mechanism governing variation for RFI across breed, diet or stages of development. Given that cattle are generally reared on high forage diets and finished on high concentrate diets, the deductions from Chapters 5 and 6 challenge, for the first time, the practicality of developing a biomarker for RFI, that would robustly predict the feed efficiency potential of an animal independent of the dietary regimen under which it was managed. Furthermore these findings suggest that the concept of nutrigenomics and 'feeding the genes' may be important to the future application of the RFI concept.

7.6 Biomarkers for RFI

A major obstacle to measuring feed efficiency in non-research settings is the need for accurate measurement of individual intake and weight gain over an extended period. This limitation is further compounded when animals are grazing as accurate measurement of daily herbage intake is not achievable. Accordingly, biomarkers have been explored as proxies for feed efficiency. Cohen-Zinder et al. (2016) have provided evidence that *FABP4* might be a suitable biomarker for RFI in Holstein cattle [353]. However, *FABP4* was not related to RFI in HF, CH or SM cattle in Chapters 5 and 6 of this thesis. Similarly, no consistency between the findings reported in this thesis and other work [182, 283, 301, 318, 337, 377, 378, 386, 387] in relation to biomarkers was observed, providing further evidence for the complex nature of this trait.

However, despite this, certain DEG identified as part of the studies outlined in Chapters 5 and 6 have previously been associated with feed efficiency and could potentially be proposed as putative biomarkers for this complex trait. In Chapter 5, *DNAJB11* and *HSPA5,* stress combat

proteins were elevated in more efficient animals, consistent with findings of [181, 287, 297, 301, 316]. Conversely, in Chapter 6, *HSPA6*, a member of the same protein family, was observed to have the opposite relationship with RFI. While these results are conflicting, the consistent appearance of these genes across multiple studies is a most interesting finding that warrants further interrogation.

The glutathione S-transferase family has been implicated in feed efficiency previously [279, 317, 329, 336, 337]. The expression of *GSTT1* in Chapter 5, is consistent with many published RFI based studies [319, 329] where upregulation of these genes in the low feed efficient animals was observed [317, 329]. *DBP* is a Protein Coding gene and among its related pathways is activation of circadian gene expression. While *DBP* has never been previously associated with feed efficiency in cattle, it has been shown that mice with increased feed efficiency have a lower expression of *DBP* [340] which is in agreement with the findings reported in Chapter 5. Finally, *SLC16A1* has been previously associated with feed efficiency [364] and in Chapter 6, expression of this gene was elevated in the less efficient animals.

This thesis has provided new information on aspects of the biochemical control of feed efficiency in beef cattle from multiple breeds managed across different dietary regimes. While there appears to be some consistency between studies, it would be naïve to label any of the genes discussed above as biomarkers for RFI without further extensive interrogation, both at the genetic and functional level.

7.7 Future work

RFI is complex and multifaceted in nature, and the findings from this thesis have highlighted some of the many areas that require additional interrogation in order to advance our understanding of the molecular mechanisms regulating the trait as well as the ultimate development of robust biomarkers for the trait.

From Chapter 3, it is evident that the contribution of mitochondria to variation for RFI is not due to differences in mitochondrial number, which is the first time that such data have been reported for cattle. Equally, our studies provide no evidence that differences in the activity of the enzymes of the electron transport chain contribute significantly to observed variation in the traits between cattle. Despite this, the precise mechanisms behind the likely relationship between mitochondria and RFI in beef cattle, as reviewed by Bottje and Carstens (2012) [375], remain to be elucidated.

When working with large animals, the logistics of sample collection are complicated and in the current thesis, in order to ensure samples were all taken at the same time period, freezing immediately was the only option available. Consequently, assays pertaining to mitochondrial ROS production, proton leak, and respiration capacity could not be performed which is a recognized limitation of the study reported in Chapter 3. Future work in this area, should focus on the measurement of the mitochondrial in a more physiologically representative state, perhaps in a similar fashion to that described by Brand et al. (2011) [388]. Such an approach would be likely to provide a better depiction of cellular mitochondria function and coupled to the results of Chapter 3 of this thesis, will give a more definitive answer to this putative quantitative relationship for beef cattle.

Five genes related to the electron transport chain were found to be related to RFI in Chapter 5. Future work, could involve western blotting and quantitative real time PCR of the respective proteins and genes in various metabolically active tissues of multiple breeds of animals to elucidate how robust in nature this relationship is.

The results of Chapter 4 highlight a strong relationship between RFI and selected molecular pathways within adipose tissue. In addition, many studies have found a positive correlation between RFI and backfat thickness [305], which could lead to unfavourable carcass changes [30, 389], should animals be selected for improved RFI in the absence of an economically

weighted multi-trait selection index based approach. There is thus a need for more studies to more comprehensively examine the molecular aspects of this relationship. For example, next generation sequencing of adipose transcripts could be undertaken in a large cohort of experimental animals of varying genetic background such as the animal model used in Chapter 6 of this thesis. Furthermore, it is well established that different adipose depots in the body have differential influence on energy homeostasis and thus feed efficiency [91] so transcriptomic and proteomic datasets from these different adipose depots would provide invaluable information pertaining to this relationship.

In Chapter 4, *GLUT4* was identified to have a robust relationship with RFI, demonstrated by its enhanced expression in both energetically efficient heifers and bulls. While *GLUT4* has not been identified by other RFI studies as a potential biomarker, the results of Chapter 4, combined with the results of Karisa et al. (2013) who predicted that biological processes associated with variation in RFI could begin with glucose uptake into the cell through *GLUT4* [283] provide adequate evidence to warrant further interrogation. *GLUT4* is the major transporter that mediates glucose uptake by insulin sensitive tissue in the body [390]. In future studies the translocation of *GLUT4* from the cytoplasm to the plasma membrane in various tissues from animals divergent for RFI, could be examined to gain a greater understanding of the functional implications related to this apparent upregulation of gene expression. An immunocytochemistry technique, using flurophore-conjugated *GLUT4*- specific antibodies such as that developed by Koshy et al. (2010) [391] could be carried out, for example. Also, an investigation looking at the protein abundance of *GLUT4* across various tissues from cattle divergent for feed efficiency could be carried out.

Chapters 5 and 6 provided evidence for DEG that are related to RFI. While, reassuringly, many of these DEG had previously been associated with RFI, the effects of RFI on gene expression were not consistent across breed, dietary phase or age in our study. The results of our study in conjunction with the corresponding data of Coyle et al. (2017) are strongly indicative that while

one of the more repeatable economically important traits, RFI status varies significantly across different dietary regimens and phases of development [62], which is not entirely unexpected. Future studies could focus more on the 50% or so of animals that maintain their RFI classification across multiple dietary phases and stages of their lives, and perform transcriptomics on key metabolic tissues in these animals such as the study conducted by Cohen-Zinder et al. (2016) [353]. The scale of such studies would, by necessity, have to be large and be representative of variation according to breed, gender and environment, however, the results would provide invaluable information regarding the biology of this complex trait. In addition, any DEG identified to be associated with RFI, in conjunction with the findings of Chapters 5 and 6 and many recent publications, are ideal candidates to be further interrogated for SNPs, which could, following appropriate validation, be incorporated into worldwide breeding programmes.

In conclusion, the main findings of this thesis can be summarized as follows:

1. Divergent phenotypic status for RFI in beef cattle was shown, for the first time, to have no effect on mitochondrial abundance in muscle or liver tissue. In addition, our studies provided no evidence for an effect of RFI status on the activity of the enzymatic complexes of the electron transport chain within skeletal muscle and liver. These findings suggest that variation in mitochondrial number and/or enzymatic capacity may not be major contributors to variation in RFI.

2. The expression of *GLUT4* in adipose tissue is related to RFI. This finding would indicate that the decreased expression of *GLUT4* and increased expression of NEFA in the high RFI animals represents less efficient glucose homeostasis and increased insulin resistance in these animals. *GLUT4* is a strong target for further interrogation as a biomarker for this trait.

3. Transcriptomic analysis in liver tissue from bulls divergent for RFI revealed evidence that efficient heifers had an enhanced capacity for mitochondrial function in muscle tissue, while

oxidative response, protein mediation and cell signaling are likely to be processes that are influencing variation in feed efficiency in bull liver tissue. Cognisance must be taken, however, of the likely physiological age/stage of development differences between the bulls and heifers employed in the study despite the fact that, chronologically, they were of similar age.

4. Efficient HF steers offered zero grazed grass subsequent to an extended period on a high concentrate diet, showed evidence of a lower immunocapacity than their less efficient counterparts as demonstrated by a dysregulation of immune system pathways. Given that the abilty to mount an immune response is metabolically costly, and, animals consuming low dry matter, zero grazed grass after a period of being accustomed to an energy dense high concentrate diet could be considered to be under a form of metabolic/nutritional stress, it is possible that the more efficient animals employ a coping mechanism by damping their immune responsiveness.

5. Effects of RFI on gene expression in muscle of beef cattle are not consistent across breed type or dietary phase. Furthermore, it is clear that there is no consistent biological mechanism governing variation for RFI across breeds, diets and various stages of development, thus challenging the practicality of development of a robust set of biomarkers for RFI that would be of ubiquitous utility to the beef cattle industry.

Chapter 8

CHAPTER 8. REFERENCES

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Chapter 9 (Appendix)

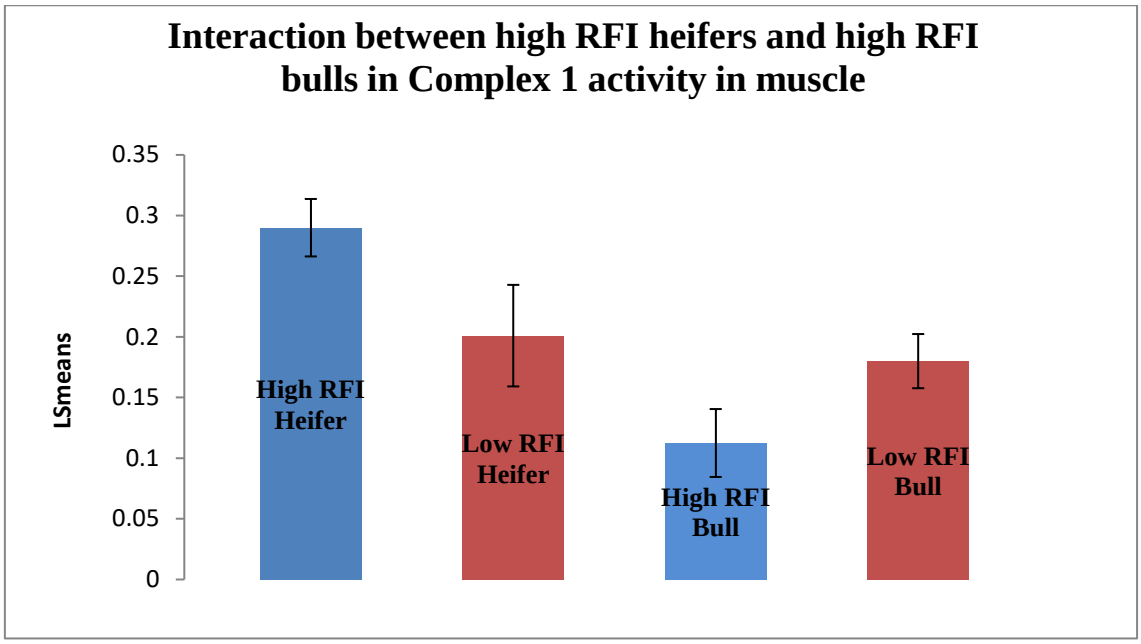

Figure 9.1. Interaction between high RFI heifers and high RFI in bulls in Complex 1 activity in muscle. HRFI = High RFI; LRFI = Low RFI; H= Heifer; B = Bull. There was a gender by RFI interaction for the activity of Complex 1 activity where high RFI heifers had a higher activity of complex 1 activity than low RFI heifers but high RFI bulls had lower activity of complex 1 than low RFI bulls.

Table 9.1. Slaughter information for heifers and bulls divergent for RFI

	Gender	Liver weight (kg)	SD	Kill weight(kg)	SD	Cold weight (kg)	SD	Kill age (months)	SD
HRFI	**Heifers**	7.12	1.14	578.67	35.89	309.96	22.33	18.89	0.87
LRFI	**Heifers**	7.15	0.90	573.20	40.03	306.02	24.57	18.30	0.90
HRFI	**Bulls**	7.68	1.20	659.14	49.30	365.15	27.76	19.09	0.79
LRFI	**Bulls**	7.66	0.81	621.75	68.85	342.95	42.32	18.63	1.22

SD = Standard Deviation
HRFI = High RFI; LRFI = Low RFI

Table 9.2 Effects of read-filtering, adapter trimming and read mapping

Sample ID	Post-filter reads (#)	Post-filter Q30 (%)	Post-filter GC (%)	STAR mapped (unique) (%)	STAR mapped (multi-) (%)	STAR failed (%)
1078P1M	45175304	98.56	49.16	91.97	7.2	0.83
1100P1M	16997499	98.96	50.24	91.32	6.83	1.86
1100P3M	33626707	98.77	51.67	91.78	7.31	0.92
1100P4M	39851602	99.31	52.6	91.41	7.56	1.02
1101P1M	52062970	99.17	51.77	91.05	7.9	1.05
1316P4M	10631313	98.46	50.35	91.05	7.86	1.09
1320P1M	16268781	99.17	52.15	91.41	7.44	1.15
1320P4M	16348763	99.16	50.8	92.2	6.9	0.9
1483P3M	29625282	98.91	51.99	91.63	7.34	1.04
1484P3M	16991758	99	54.36	91.45	7.44	1.1
1484P4M	15249193	99.3	55.77	69.62	25.54	4.84
1492P3M	7574617	98.89	53.68	92.52	6.59	0.89
1492P4M	16646388	99.15	52.31	91.79	7.2	1.02
1639P3M	55798606	98.61	50.47	91.41	7.14	1.44
1639P4M	25025417	99.33	49.4	91.56	7.54	0.89
1643P1M	11332762	99.35	51.42	92.15	6.85	1
1656P1M	25806308	98.77	49.98	91.09	7.8	1.11
1657P4M	15842574	99.12	51.01	92.63	6.46	0.91

P= Phase; M = Muscle; Q30 = Q-score where one base call in 1,000 is predicted to be incorrect; GC = guanine cytosine content; STAR = Spliced Transcripts Alignment to a Refer

Table 9.2 (contd). Effects of read-filtering, adapter trimming and read mapping

Sample ID	Post-filter reads (#)	Post-filter Q30 (%)	Post-filter GC (%)	STAR mapped (unique) (%)	STAR mapped (multi-) (%)	STAR failed (%)
1661P4M	27357811	99.01	50.65	92.49	6.74	0.77
1668P3M	13985897	98.79	55.87	89.24	9.08	1.68
1782P3M	5767635	98.98	48.5	92.97	6.31	0.71
1797P1M	15070004	98.83	50.9	91.3	7.78	0.92
1811P1M	17689773	98.51	50.58	91.84	7.19	0.97
1811P4M	21981222	98.67	54.34	91.83	6.95	1.22
1816P1M	20895478	99.11	52.04	90.84	7.83	1.34
1816P3M	31797705	98.75	51.41	90.5	8.41	1.1
1831P1M	19255783	98.68	51.08	89.72	9.03	1.25
1831P3M	15675547	99.31	54.31	92.11	6.79	1.11
1840P4M	18648946	99.2	55.08	77.19	19.38	3.43
1843P3M	5453451	99.25	56.38	90.94	7.85	1.21
1844P4M	27397989	98.85	51.35	91.72	7.38	0.9
1846P1M	10731017	99.23	54.09	90.1	8.64	1.25
1877P1M	34964705	99.25	51.36	91.16	7.6	1.24
1879P3M	31548798	99.34	51.07	91.66	7.35	0.99
1909P3M	40109	99.27	50.1	91.97	7.15	0.88

Table 9.2 (contd). Effects of read-filtering, adapter trimming and read mapping

Sample ID	Post-filter reads (#)	Post-filter Q30 (%)	Post-filter GC (%)	STAR mapped (unique) (%)	STAR mapped (multi-) (%)	STAR failed (%)
2297P4M	12262978	99.28	53.16	93.82	5.23	0.96
307P1M	45762661	99.26	50.66	91.95	7.01	1.04
307P3M	30854450	99.02	51.81	92.24	6.74	1.02
307P4M	19670743	99.06	54.24	92.59	6.42	0.99
310P3M	23007425	99.14	50.82	90.06	8.68	1.27
310P4M	21894947	98.92	52.43	91.98	7.18	0.83
318P3M	22967787	99.32	49.52	92.12	6.83	1.05
322P1M	18617184	99.37	51.44	91.62	7.43	0.95
322P4M	19576636	99.15	49.82	92	7.02	0.98
357P3M	5247	98.71	51.49	91.01	7.3	1.68
364P1M	25261003	99.32	51	90.87	7.89	1.24
367P4M	6729139	98.8	51.67	91.66	7.11	1.24
419P4M	20587111	98.35	50.13	91.65	7.6	0.75
427P3M	23428151	99.24	51.87	91.51	7.66	0.82
437P3M	12019560	99.31	52.5	91.31	7.66	1.03
443P1M	14072282	99.1	53.62	90.19	8.44	1.37
443P4M	16018425	99.14	49.41	91.24	7.7	1.06
447P1M	30405225	98.97	53.2	89.42	9.16	1.42
449P1M	41005187	99.2	53.17	89.74	8.89	1.37
449P3M	34516260	99.3	52.25	91.63	7.52	0.84

Table 9.2 (contd). Effects of read-filtering, adapter trimming and read mapping

Sample ID	Post-filter reads (#)	Post-filter Q30 (%)	Post-filter GC (%)	STAR mapped (unique) (%)	STAR mapped (multi-) (%)	STAR failed (%)
2064P1M	18295708	99.39	50.09	91.38	7.62	1
2064P3M	8307329	98.67	50.14	94.72	4.65	0.62
2065P1M	16776839	98.79	55.13	90.86	7.83	1.31
2065P3M	20529704	98.44	53.43	91.62	7.2	1.18
2068P1M	30930425	99.2	51.85	90.46	8.26	1.29
2069P1M	24030994	98.73	51.77	91.54	7.43	1.03
2069P3M	66330552	99.36	50.65	91.3	7.43	1.26
2071P1M	36644140	98.86	54.28	88.02	10.42	1.56
2071P4M	47408647	99.28	51.92	92.83	6.29	0.88
2085P3M	32641591	99.13	51.76	89.93	8.57	1.5
2087P3M	5136281	98.53	48.45	91.63	7.46	0.91
2094P3M	20706480	99	52.31	91.31	7.63	1.05
2095P1M	22738077	99.03	50.89	91.44	7.61	0.95
2095P4M	23266157	99.03	51.82	91.73	6.87	1.41
2102P1M	14751077	99.39	55.67	51.64	40.17	8.19
2220P1M	23429866	99.1	50.75	91.67	7.51	0.82
2220P4M	27504977	99.22	52.12	91.48	7.21	1.31
2251P1M	134611	98.6	51.19	91	7.85	1.15
2259P1M	5741859	99.11	50.82	92.43	6.59	0.99
2266P4M	36624783	97.61	51.58	91.73	7.42	0.86
2272P3M	21742705	99.03	50.52	91.85	6.97	1.17

Table 9.2 (contd). Effects of read-filtering, adapter trimming and read mapping

Sample ID	Post-filter reads (#)	Post-filter Q30 (%)	Post-filter GC (%)	STAR mapped (unique) (%)	STAR mapped (multi-) (%)	STAR failed (%)
5632P4M	8708	97.89	49.92	91.76	7.41	0.83
5633P3M	17950069	99.18	53.29	92.13	6.77	1.1
5635P1M	24011015	99.11	50.77	91.24	7.66	1.1
5635P3M	15623884	97.67	49.94	91.36	7.5	1.14
571P3M	19691469	99.21	46.42	92	7.18	0.82
581P1M	40638121	99.37	50.95	91.37	7.53	1.11
718P4M	22326434	99.37	52.37	91.42	7.46	1.12
723P1M	26005017	99.13	51.28	92.07	6.99	0.94
788P3M	43344893	98.77	52.56	90.82	8.03	1.14
854P1M	19364526	99.33	53.79	92.32	6.53	1.15
856P1M	71334271	99.13	50.37	91.5	7.5	0.99
856P4M	2566019	99.05	53.03	93.8	5.06	1.14
871P4M	747659	99.44	46.59	92.62	6.54	0.83
954P4M	194038	99.28	50.64	90.45	8.52	1.04

Table 9.2 (contd). Effects of read-filtering, adapter trimming and read mapping

Sample ID	Post-filter reads (#)	Post-filter Q30 (%)	Post-filter GC (%)	STAR mapped (unique) (%)	STAR mapped (multi-) (%)	STAR failed (%)
5588P4M	16458508	98.75	48.08	91.52	7.51	0.97
5605P3M	15380701	97.29	49.44	92	7.1	0.89
5609P4M	25571229	98.64	52.09	92.73	6.46	0.81
5612P3M	32433766	99.01	53.19	91.89	6.96	1.15
5612P4M	14782113	99.1	49.48	92.37	6.8	0.83
5613P1M	21786374	97.67	52.74	89.47	9.12	1.42
5613P3M	20631509	99.38	50.99	91.18	7.73	1.1
5616P3M	29124727	99.07	50.62	91.19	7.02	1.8
5617P4M	19277098	97.12	48.71	90.65	8.29	1.05
5624P1M	15405268	98.66	53.77	91.65	7.16	1.2
5632P1M	12858959	99.11	53.6	92.88	6.12	1
5632P3M	39806410	98.69	53.76	92.98	6.14	0.88

P= Phase; M = Muscle; Q30 = Q-score where one base call in 1,000 is predicted to be incorrect; GC = guanine cytosine content; STAR = Spliced Transcripts Alignment to a Reference