Genomic and Transcriptomic Investigations into the Feed Efficiency Phenotype of Beef Cattle

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Declaration

I declare that this thesis has not been submitted as an exercise at this or any other university.

I declare that this thesis is entirely my own work, except where otherwise stated.

Signed:

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Marc Higgins

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Abstract

Improving the feed efficiency of cattle is a method to increase profits while simultaneously reducing the environmental impact of beef production. Residual feed intake (RFI) is a measure of feed efficiency, calculated as the difference between actual and predicted feed intake. Low-RFI (feed efficient) cattle consume less feed than their high-RFI (inefficient) counterparts while maintaining their growth rate and emitting less methane. Therefore, incorporation of RFI into breeding programmes represents an opportunity to improve profitability while reducing the environmental impact of beef production. However, RFI is a difficult trait to measure, requiring expensive and time consuming feeding trials. Identification of biomarkers for RFI would enable genomic-assisted selection for this trait, circumventing the need for continual feeding trials. There is considerable variability associated with RFI whereby cattle have been observed to re-rank in terms of this trait when offered varying diets and different breeds display inherent variation in RFI. Consequently, robust biomarkers for RFI must be identified which are applicable across breed and diet. The difficulty in identifying robust biomarkers for this trait has been an impediment to the adoption of RFI in genomic assisted breeding programmes. The aims of this thesis were: (i) To identify SNPs associated with RFI in a multi-breed and crossbred reference population of Irish beef cattle for inclusion in the Irish genomic assisted breeding programme. (ii) To identify differentially expressed genes associated with RFI-divergence in two breeds of steers offered three diets throughout their lifetime, which may be candidate genes for interrogation for the discovery of biomarkers for RFI. (iii) To identify key regulatory genes and biological processes associated with variation in RFI based on gene expression data across two breeds and three dietary phases, which may be candidates for further work while enhancing our understanding of the biology underlying RFI-divergence.

In my first study, seven, fourteen and three SNPs associated with RFI, average daily gain and feed intake, respectively, were identified in a multi-breed reference population of Irish beef cattle (n = 1,492). To investigate the effects of associated variants on nearby genes, expression quantitative trait loci (eQTL) analysis was carried out. One eQTL, between rs43555985 and *GFRA2*, was identified for RFI. rs43555985 was a SNP associated with RFI following meta-analysis.

Following this, RNA-Seq analysis was performed to investigate the hepatic transcriptome of Charolais and Holstein-Friesian steers divergent for RFI. These steers were offered three differing diets throughout their lifetime. A total of 355 differentially expressed genes were identified across all diet-breed combinations. Three genes, *GADD45G, HP* and *MID1IP1*, were differentially expressed across two dietary phases for the Charolais steers. No gene was differentially expressed across all three dietary phases for either breed, however several physiological processes such as immune function and lipid metabolism, were enriched across all diet and breed combinations.

In order to gain insight into gene networks and key regulatory genes implicated in RFI-divergence, weighted gene co-expression network analysis (WGCNA) of the RNA-Seq data was carried out. WGCNA allows identification of modules of genes associated with RFI. These modules are genes which have similar expression profiles and may work in unison to affect the phenotype. Similarly, WGCNA can identify hub genes which are postulated to be master regulators of gene expression within their modules. These genes and modules may be candidates for further study in order to identify SNPs associated with RFI across diet and breed. This analysis identified a total of ten modules significantly associated with RFI. Within these modules, 349 hub genes were identified. Thirty-seven of these have previously been associated with feed efficiency in livestock while ten hub genes were identified as differentially expressed in the earlier RNA-Seq analysis. Hub genes were found to play roles in protein turnover and mitochondrial efficiency amongst other physiological processes.

Overall, these genomic and transcriptomic analyses have provided insight into the biology of feed efficiency and can form the basis of biomarker discovery for genomic selection for RFI. Results reported within this thesis support previous findings with regards to the role of physiological processes such as lipid metabolism and protein turnover in RFI-divergence. This thesis also reports novel findings such as the first eQTL identified for RFI in beef cattle. These results together have utility to inform future genomic assisted breeding programmes.

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List of Abbreviations

AA	Aberdeen Angus
ACTH	Adrenocorticotropic hormone
ADG	Average daily gain
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
BB	Belgian Blue
B-H	Benjamini-Hochberg
bp	Base-pair
BW	Body weight
CCK	Cholecystokinin
cDNA	Complementary DNA
CH	Charolais
Chr_mb	Chromosome_megabase
CNS	Central nervous system
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DEG	Differentially expressed gene
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EBI	Economic breeding indexes
eQTL	Expression quantitative trait loci
FCE	Feed conversion efficiency

FCR	Feed converstion ratio
FE	Feed efficiency
FI	Feed intake
g	Gram
GEBV	Genomic estimated breeding value
GIT	Gastrointestinal tract
GS	Gene Significance
GWAS	Genome-wide assocation study
H1	High-concentrate, phase 1
H2	High-concentrate, phase 2
HD	High density
HF	Holstein-Friesian
HPA	Hypothalamic-pituitary-adrenal
ICBF	Irish Cattle Breeding Federation
IDB	International dairy and beef
IGF-I	Insulin-like growth factor-I
IPA	Ingenuity Pathway Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilogram
LD	Linkage disequilibrium
LM	Limousin
MAF	Minor allele frequency
MBW	Metabolic body weight

ml	Millilitre
MM	Module Membership
mRNA	Messenger RNA
NEFA	Non-esterified fatty acids
ng	Nanogram
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
PEG	Partial efficiency of growth
QC	Quality control
QTL	Quantitative trait loci
RFI	Residual feed intake
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
ROS	Reactive oxygen species
SBS	Sequencing-by-synthesis
SE	Standard error
SI	Simmental
SNP	Single nucleotide polymorphism
ssGWAS	single-step GWAS
SVS	SNP Variation Suite
TOM	Topological overlap matrix
UTR	Untranslated region
VFA	Volatile fatty acids

VST	Variance stabilizing transformation
WGCNA	Weighted gene co-expression network analysis
WGS	Whole genome sequencing
ZG	Zero-grazed grass
βHB	Beta-hydroxy butyrate
µl	Microlitre

Chapter 1

Introduction

1. Introduction

1.1. The increasing global demand for beef

Global agriculture faces huge challenges in order to feed the ever expanding global population (Gill et al., 2018). According to the United Nations the global population is expected to increase by 32% by 2050 (United Nations, 2015). In order to meet the food demands of this growing population, global food production will need to double over the next 30 years. Growth of the middle-classes in developing countries will lead to a shift towards more calorie dense diets. This will lead to increased meat consumption (Foley et al., 2011). In order for Ireland to benefit as fully as possible from this increased demand two main criteria must be taken into consideration. First, agriculture must attempt to reduce its environmental impact while increasing production (Wollenberg et al., 2016). Secondly, farmers must find ways to maximise profits from beef production. As farmers have no control over the price charged to the consumer, input costs must be decreased to increase profits (Arthur and Herd, 2008). In Ireland, 90% of all beef produced is exported, contributing approximately €1.9 billion to the Irish national economy. This makes Ireland the largest net exporter of beef in the European Union (EC, 2014). This suggests that Ireland is well placed to meet the growing global demand for beef. However, any intensification of production must be carried out in a sustainable manner.

1.2. Irish beef production

In Ireland, there are approximately 2.3 million cows, with 1.1 million of those suckler cows on beef production enterprises (CSO, 2016). These suckler-bred beef production enterprises account for 50% of Irish beef output. The sustainability of these systems is crucial to the Irish beef industry and consequently the Irish economy. Ireland's beef production system is predominately grass based, with over 70% of suckler calf births occurring between February and May (DAFM, 2016). These calves are sired by predominately Charolais, Simmental, Limousin or Belgian Blue bulls (DAFM, 2016). However, with the removal of Irish milk quotas there has been an increase in male dairy calves incorporated into beef production systems (Murphy et al., 2017). These births are timed to coincide with grass growth, and animals graze outside for up to eight months before they are housed indoors. Once

cattle are moved indoors, their diet changes from fresh grass to a combination of grass silage and concentrates (O'Donovan et al., 2011). Cattle are then moved outside in spring to graze on grass which is a more economic feedstuff than grass silage or concentrates (Finneran et al., 2010).

Despite the abundance of grass in Ireland, feed accounts for approximately 75% of input costs on beef production enterprises (Finneran et al., 2010). Therefore, any reduction of expenditure on feed will lead to increased farm profits, particularly if output is maintained (Herd et al., 2003). The incorporation of feed efficient cattle, i.e. cattle that efficiently convert feed to produce (sellable meat), into the Irish national herd is one mechanism by which farm profits could be increased while maintaining output levels. However, the identification of feed efficient cattle is a long and expensive process. Therefore the identification of biomarkers to enable fast, cost effective selection of feed efficient cattle is of great importance.

1.2.1. How can Irish beef meet increasing global food demands

Traditionally, if increased agricultural output was required, farmers would increase herd size and total area farmed. However, increasing the size of the national herd would lead to increased methane emission, leading to Ireland failing to meet European and International agreements on climate change (EC, 2014, Dace and Blumberga, 2016). Annually, ruminants are responsible for 14% of methane released into the atmosphere (Huws et al., 2018). It has been suggested animals with greater feed efficiency (FE) emit less methane than their non-efficient counterparts (Fitzsimons et al., 2013). Therefore, incorporation of feed efficient animals into the national herd may reduce national greenhouse gas emissions.

The second traditional method of intensifying agricultural practices is by increasing land used for agriculture (Garnett, 2009). There is now a global drive to minimise agricultural land-use in order to slow climate change. Deforestation is often directly due to the increased need for agricultural land and is a major contributor to global warming (Foley et al., 2011). Decreasing land use associated with agriculture is a

potential method to reduce the environmental impact of agriculture. Incorporation of animals that efficiently convert feed to produce will require less pasture and improve the sustainability of agriculture (Broderick, 2017, Cantalapiedra-Hijar et al., 2018). However, as FE is a difficult trait to measure quickly and accurately, identification of suitable biomarkers for FE is desirable. This could enable rapid and cost-effective selection for FE via genomic assisted breeding programmes (Kenny et al., 2018).

1.3. Feed efficiency and its measures

Due to the importance of FE to beef production systems several measures of this trait exist, each differing in their methods of calculation and application (Berry and Crowley, 2013). These measures include feed conversion ratio (FCR) and its inverse feed conversion efficiency (FCE), partial efficiency of growth (PEG) and residual feed intake (RFI) (Arthur et al., 2001, Crews Jr, 2004, Herd et al., 2003). These methods of quantifying FE are summarised in Table 1.1.

Traditionally, feed conversion ratio (FCR) has been widely used as a measure of FE in beef production systems. However, FCR is negatively correlated with growth rate and mature size (Crews Jr, 2004). Therefore, selecting animals for more desirable (decreased) FCR leads to selection of larger animals with increased maintenance requirements.

RFI has become the preferred measure of FE (Savietto et al., 2014, Kenny et al., 2018, Cantalapiedra-Hijar et al., 2018). Selection of animals based on RFI presents two key advantages. First, RFI is moderately heritable with a heritability range of 0.07-0.62 (Berry and Crowley, 2013). Therefore, selecting animals based on RFI enables their genetics to be maintained and propagated through the herd. Secondly, when cattle are fed *ad libitum*, RFI is phenotypically independent of growth traits and their levels of production (Kenny et al., 2018, Berry and Crowley, 2013). This is a desirable characteristic as selection of low-RFI (i.e. high FE) cattle will result in maintenance of output and an increase in profits. This is because low-RFI cattle

consume less feed than high-RFI cattle while displaying the same levels of output. This results in less expenditure on feed, thus improving profits. In this thesis the focus will be on RFI and processes contributing to variation in this trait.

Table 1.1 Definition of traits used to measure feed efficiency in beef cattle

Trait	Definition	Formula	Reference
FCR	Ratio of feed intake to weight gain	DMI ÷ ADG	Crews Jr (2004)
FCE	Ratio of weight gain to feed intake	ADG ÷ DMI	Crews Jr (2004)
PEG	Ratio of weight gain to feed intake after expected requirements for maintenance have been subtracted	ADG ÷ (DMI – DMIm), where DMIm = expected DMI as derived from feeding standard formulae	Arthur et al. (2001)
RFI	Difference between an animal's actual feed intake and predicted feed intake based on its requirements for growth and body size	Actual DMI – predicted DMI, where predicted DMI is derived from regression of feed intake on ADG and average $BW^{0.75}$	Berry and Crowley (2013)

FCR = feed conversion ratio; FCE = feed conversion efficiency; PEG = partial efficiency of growth; RFI = residual feed intake; DMI = dry matter intake; ADG = average daily gain; BW = body weight. Adapted from Berry and Crowley (2013) and Archer et al. (1999).

1.3.1. Residual feed intake

RFI was first described as a measure of FE in growing cattle in 1963 (Koch et al., 1963) and is defined as the difference between an animal's actual feed intake minus its predicted feed intake (Figure 1.1). The amount remaining, or the residual, reflects that animal's FE (Koch et al., 1963, Arthur and Herd, 2008). Animals that consume less feed than expected for their size and body weight over a defined recording period would have a negative residual and are termed low-RFI and are considered to be highly FE. The inverse is also true in that animals which consume more feed than expected have a positive residual, are deemed to have high-RFI and are lowly feed efficient (Crews Jr, 2004).

Figure 1.1. An example of the regression line used to calculate RFI. Each data point represents an actual DMI measurement for an individual animal, while the solid regression line represents the predicted DMI for this group of cattle at varying levels of production. The difference between the measured DMI and the predicted DMI for any one animal represents their RFI. Animals with data points above the regression line are high-RFI cattle and are deemed to be inefficient as they consume more than their predicted DMI. Adapted from Sainz and Paulino (2004).

1.3.2. Statistical calculation of RFI

Calculation of an animal's RFI involves predicting its feed intake, most commonly by linear regression of actual feed intake measurements. Using linear regression to calculate an animal's predicted feed intake makes RFI independent of production traits, namely average daily gain (ADG) and metabolic body weight (MBW) (Crowley et al., 2010), and enables comparison of individuals differing in production level (Arthur and Herd, 2008). As RFI is independent of production traits, it has been highlighted as a useful mechanism to investigate the inherent biological variation leading to FE (Berry and Crowley, 2013). The linear regression equation used to calculate predicted feed intake, as predicted by dry matter intake (DMI), of an individual animal can be summarised as:

$$y = \beta_0 + \beta_1(ADG) + \beta_2(MBW) + RFI$$

Where y is DMI, β_0 is the regression intercept, β_1 is the partial regression of daily feed intake on ADG and β_2 is the partial regression of daily feed intake on MBW. MBW is calculated as an animal's body weight to the power of 0.75.

1.3.3. RFI measurement trial

In order for RFI to be calculated, individual measurements of feed intake and daily gain must be taken for each animal throughout a long-term feeding trial. The required duration of the RFI trial varies throughout the literature with some authors reporting required trial lengths as long as 120 days with others reporting trials as short as 56 days, with regular and automated weighing (Archer et al., 1999, Kearney et al., 2004). The current standard suggested trial length for calculation of RFI is 70 days (Cundiff et al., 2010).

Prior to commencement of the trial, animals must be allowed an acclimatisation period of 21 days to allow adaptation to the diet offered and the facilities within which the trial is to be carried out. Once the trial begins, animals must be weighed twice on consecutive days at the start of the trial with this consecutive weighing also occurring at the end of the trial. During the trial live weight measurements must be taken periodically, and feed intake must be accurately quantified (Cundiff et al., 2010).

During the trial, animals must be allowed individual access to *ad libitum* feed. This is to ensure accurate measurements of individual DMI, and the diet offered must ensure that the animal has adequate energy and roughage for rumen function. Furthermore, to allow comparison of FE across animals within a trial, only one diet should be offered during a test period. This is important as animals may consume differing amounts of feed depending on the physical characteristics or energy content of the diet offered leading to variation in DMI across diets offered. As DMI is a key component of RFI, any introduced variation in DMI may prevent accurate calculation of RFI (Cundiff et al., 2010, McDonald, 2002).

1.3.4. Effect of breed on RFI

As well as animal size and growth rate, breed plays a role in variation of RFI. Previous work investigating the efficiency of Irish beef bulls of different breeds offered an *ad libitum* concentrate diet, found Charolais and Limousin bulls to be the most efficient, while Aberdeen Angus and Hereford bulls were the least efficient (Crowley et al., 2010). Similarly Schenkel et al. (2004) found that Limousin bulls were most efficient, while Aberdeen Angus were the least efficient. It can be concluded from these studies, and others, that within the primary beef breeds present in Ireland the most efficient are Limousin and Charolais, while Simmental and Aberdeen Angus are the least efficient (Schenkel et al., 2004, Crowley et al., 2010, Clarke et al., 2009). Dairy breeds are considered to be less efficient than beef breeds (Keane, 2011). However, crossing dairy cows with bulls from late maturing beef breeds such as Charolais and Limousin improves the efficiency of their offspring (Huuskonen et al., 2013).

1.3.5. Heritability of RFI

RFI has been suggested to be a moderately heritable trait, with an estimated average heritability of 0.33 (Berry and Crowley, 2013). It has been shown that offspring of a low-RFI sire grew 19% more rapidly than progeny of a high-RFI steer with no increase in feed intake while also having a 26% lower RFI ranking (Arthur and Herd, 2008). Dam RFI has also been illustrated to impact on the RFI of their progeny whereby low-RFI dams produced low-RFI offspring while offspring of high-RFI dams were also classified as high-RFI (Basarab et al., 2007). The fact that RFI is a heritable trait suggests that incorporation of RFI in breeding programmes will lead to gains in FE that will be retained and maintained through cattle populations (Kenny et al., 2018).

1.3.6. Repeatability of RFI in beef cattle

For RFI as a trait to have utility as a selection tool for FE, it must be consistent across the various stages of an animal's life and the diets which may be offered to that animal. It has been suggested that there is a genotype-by-environment

interaction when animals are offered diets varying in energy density (Cassady et al., 2016). This is of particular importance for breeding programmes as animals must be selected for performance early in life (Lawrence et al., 2012b). Moreover, repeatability of RFI across diet is important in beef cattle production systems where animals may be offered multiple diets throughout the production cycle.

It has been illustrated that, when offered the same diet across two periods, RFI is moderately repeatable (Cassady et al., 2016, Kelly et al., 2010b). Cassady et al. (2016) has illustrated that when beef steers are fed a grain based diet across two periods, RFI has a repeatability of r = 0.63, *P*-value < 0.05, while Kelly et al. (2010b) observed that beef heifers offered a high concentrate diet across two periods also demonstrate repeatable levels of RFI, r = 0.62, *P*-value < 0.001. However, Durunna et al. (2011a) reported that RFI was less repeatable (r = 0.33, *P*-value <0.001) when beef steers were offered a grower diet, followed by a finisher diet. Meyer et al. (2008) and Lawrence et al. (2012a) observed that heifers and cows ranked as high- or low-RFI during an indoor feeding trial did not exhibit any statistically significant differences in body weight gain or intake when returned to pasture. However, authors of these studies have noted the difficulties in measuring consumed feed for grazing cattle as a possible limitation in detecting statistically significant differences in intake between groups (Lawrence et al., 2012a, Meyer et al., 2008). Conversely, Manafiazar et al. (2015) observed that heifers ranked as low-RFI following a high-concentrate indoor feeding trial consumed significantly less grass at pasture than their high-RFI counterparts. In a recent study investigating repeatability of RFI across dietary phases of beef steers, Coyle et al. (2017) found that 40% of cattle maintained their RFI classification (i.e. high-, medium- or low- RFI) when RFI was calculated first for a high-concentrate dietary phase, followed by a zero-grazed grass dietary phase.

As it is clear that some amount of re-ranking occurs across dietary stages, this suggests that there is a genotype-by-environment interaction for RFI (Durunna et al., 2011b, Kenny et al., 2018). Re-ranking of cattle with regards to RFI when the diet offered differs represents a challenge in uncovering biomarkers for RFI. Any

potential biomarkers proposed for RFI must be applicable across diet to have maximum utility.

1.4. Physiological basis for RFI variation

As RFI is mathematically independent of the level of production for the animal it is calculated for, it has been suggested that variation in RFI represents inherent biological variation in FE (Herd and Arthur, 2009, Kenny et al., 2018). The biological networks which lead to variation in RFI have yet to be fully uncovered. However, several mechanisms are hypothesized to impact upon RFI and other measures of FE. It has been suggested that the main mechanisms contributing to altered FE include feed intake, digestion of consumed feed, metabolism, body composition and activity levels (Fitzsimons et al., 2017) (Figure 1.2).

1.4.1. The impact of feeding behaviour on RFI

Feed intake is regulated by interactions between physiological drivers of feed intake such as hormones and physical properties of feed (Allen, 2014). However, there are factors influenced by feed intake that may impact upon FE. For example, the activity

associated with consumption affects divergence in FE. Fitzsimons et al. (2014b) observed that high-RFI cows spent a longer duration feeding when compared to their low-RFI counterparts while they also consumed more feed, which was consistent with Gomes et al. (2013) who observed that low-RFI Nellore steers spent less time feeding than high-RFI steers. Similarly to time spent feeding, it has been noted that low-RFI cattle have fewer feeding events than their high-RFI counterparts (Kelly et al., 2010b). These findings are in agreement with Herd et al. (2004) who estimated that the variation in activity and feeding in RFI divergent cattle could contribute up to 5% of the observed difference in RFI.

1.4.2. Activity levels and feed efficiency

Variation in activity between animals may result in differences in energy expenditure, and consequently variation in energy available for maintenance and growth (Herd and Arthur, 2009). When investigating the effect of locomotion on RFI, Richardson et al. (1999) observed a positive correlation between RFI and locomotion as measured by pedometer count. Similarly, Llonch et al. (2018) observed that more active steers displayed lower FE than their less active counterparts. In contrast, Lawrence et al. (2012a) and Lawrence et al. (2011) found no difference in activity levels in RFI-divergent beef cows and heifers, respectively, when activity levels were measured in pens. However, when these cows were released to pasture it was noted that high-RFI cows spent more time standing than low-RFI cows.

1.4.3. Appetite and RFI

Appetite has also been suggested as a source of variation in RFI (Lines et al., 2014). Appetite is a complex drive, with many components integrated by the central nervous system (CNS) (Allen (2014), Figure 1.3). Orexigenic signals increase hunger while anorexigenic signals increase satiety (Allen, 2014). The arcuate nucleus (ARC) in the hypothalamus is a major site of appetite regulation (Roche et al., 2008). Leptin, an example of an anorexigenic hormone produced primarily by adipose cells (Zieba et al., 2005), acts on receptors within the ARC to prevent secretion of orexigenic

peptides such as neuropeptide Y and agouti-related protein (Roche et al., 2008, Elias et al., 1999). Ghrelin, an example of an orexigenic hormone secreted by the abomasum in ruminants, causes the release of orexigenic peptides from the ARC (Wertz-Lutz et al., 2006). Ghrelin is released when there is a negative energy balance, but once intake and expenditure are balanced ghrelin secretion decreases (Bagnasco et al., 2002).

Figure 1.3. Hormonal regulation of appetite as integrated by the hypothalamus. "+" indicates that a secreted hormone increases appetite, while "-" indicates an anorexigenic signal, CCK = cholecystokinin. Adapted from Fitzsimons et al. (2017).

Expression levels of leptin, an anorexigenic hormone, have been observed to be increased in adipose tissue (Perkins et al., 2014) and skeletal muscle of low-RFI cattle (Mota et al., 2017). However, leptin levels in the blood of RFI-divergent cattle have not been found to reflect RFI status (Kelly et al., 2010a). Circulating levels of ghrelin have been associated with lower FE as measured by gain-to-feed ratio (Foote et al., 2014). The hypothalamus plays an integral role in the control of appetite (Roche et al., 2008), consequently investigation into gene expression in this brain region has been conducted to examine its role in RFI variation. For example, Perkins et al. (2014) noted that hypothalamic expression of orexigenic genes is decreased in

low-RFI cattle, while levels of a satiety inducing gene, *POMC*, is increased. Contrary to this however, Reyer et al. (2018) did not observe any statistically significant differences in the expression of satiety related genes in the hypothalamus of RFI-divergent pigs. These results collectively suggest a potential role for hypothalamic genes in RFI variation, however further work is required to elucidate the exact mechanism of hypothalamic control on RFI.

1.4.4. Digestion of consumed feed and the role of the rumen in RFI

Digestion of feed in the rumen determines the nutrients available to cattle (Dijkstra et al., 2007). Feed consumed by ruminants is first broken down physically, by mastication, followed by rumination. After rumination, the broken down feed, which cannot be digested by the animal, is fermented anaerobically by microbial populations in the rumen (Janssen, 2010). The primary end products of rumen fermentation are volatile fatty acids (VFAs). VFAs are absorbed across the ruminal wall and are a major source of energy for the ruminant (Krehbiel, 2014).

The level of digestion of consumed feed is determined by the time for which feed is retained in the rumen, which in turn is determined by the rate of passage of feed into the rumen. As more feed is consumed, relative to an animal's maintenance level, total diet digestibility decreases (Huhtanen et al., 2006). From this it would follow that high-RFI cattle would exhibit lower diet digestibility. Furthermore, genetic variation in ability to digest feed has been suggested as a contributory factor to RFI variation (Herd and Arthur, 2009). Several studies have investigated the role of variation in digestibility as a potential source of phenotypic differences in RFI, and there has been conflicting findings. For example, Fitzsimons et al. (2013) and Lawrence et al. (2013) reported that there was no statistically significant difference in digestion between high- and low-RFI cattle. While Richardson et al. (2004) and Bonilha et al. (2017) reported that low-RFI cattle have greater ability for digestion than high-RFI cattle.

However, as it is difficult to accurately measure minute differences in diet digestibility, caution should be observed when attributing divergence in RFI to variation in digestive processes (Herd and Arthur, 2009). Basarab et al. (2013) suggested that the observed differences in feed digestibility between RFI-divergent cattle may truly result from differences in feed intake rather than inherent variation in an animal's ability to digest feed.

While it has been suggested that rumen fermentation accounts for a proportion of variation in RFI (Herd and Arthur, 2009), published literature reports conflicting findings regarding the difference in rumen fermentation measurables, such as pH and the concentration of VFAs, between RFI divergent cattle. Fitzsimons et al. (2014a) found no difference in rumen pH in bulls divergent in RFI, similarly McDonnell et al. (2016) who investigated rumen fermentation profiles of RFI divergent heifers observed no difference in rumen pH between RFI groups. Investigation into the concentration of VFA in the rumen digesta of high- and low-RFI cattle has not led to a definitive conclusion. Studies in Irish cattle have not found any differences in VFA concentrations between cattle divergent in RFI (Fitzsimons et al., 2014a, Fitzsimons et al., 2014b, Fitzsimons et al., 2013). However, increased expression of genes involved in absorption of VFAs across the rumen wall was observed in two populations of low-RFI cattle, leading the authors to conclude that RFI divergent cattle may differ in their ability to absorb VFAs for use as an energy source (Kong et al., 2016b, Elolimy et al., 2018).

1.4.5. Methane production and RFI

Methane is produced in the rumen by methanogens, which use hydrogen gas as their primary energy source. Methane, which cannot be absorbed or otherwise utilised by the animal, is consequently released into the atmosphere (Janssen, 2010). This generation of methane represents a loss of energy from the ruminant (Pickering et al., 2015) as well as a major source of greenhouse gas emissions, thus contributing to global warming (Huws et al., 2018). Feed intake and methane production have been shown to be positively correlated. For example, Herd et al. (2014) observed a

correlation of r = 0.65 between DMI and methane production for Angus cattle offered a high roughage diet. Similarly, Bird-Gardiner et al. (2017) observed that methane production also increased with DMI for both roughage-based and grain-based diets, r = 0.75 and 0.62, respectively. Consequently, it would be expected that low-RFI cattle, who consume less feed than high-RFI cattle, emit less methane. It has been observed that low-RFI Simmental heifers offered grass silage emitted less methane than their high-RFI counterparts (Fitzsimons et al., 2013). These authors also suggested that an increase in DMI of 1kg dry matter/day was associated with a 23g/day increase in methane emissions (Fitzsimons et al., 2013). Similarly, a positive relationship between RFI and methane emissions has been noted for Angus steers offered a grain based diet (Hegarty et al., 2007). However, others report no statistical differences in methane emissions between RFI-divergent cattle. For example, McDonnell et al. (2016) found that no differences in methane production were present between RFI-divergence Limousin-Holstein-Friesian crossbred heifers offered three different diets. However, these authors suggested that the greater diet digestibility displayed by the low-RFI heifers may contribute to the observed variation in methane emissions. Pickering et al. (2015) has suggested that selection for low RFI cattle in breeding programmes may reduce methane emissions by 11-26% in ten years, highlighting the potential environmental importance of selecting for low-RFI cattle. While the selection of low-RFI cattle likely has utility to reduce the environmental impact of beef production, further work is required to ensure there are no undesirable effects of RFI selection on methane emissions.

1.4.6. Body composition and its effect on RFI

Body composition refers to the level of fatness or leanness of an animal's carcass (Kenny et al., 2018). Body composition, as well as being of importance to the value of beef cattle, plays a major role in overall energy homeostasis (Fitzsimons et al., 2017). In terms of energy cost, it is much more expensive to deposit fat than protein, while maintenance of body protein requires greater energy use (Welch et al., 2012). Due to variation in the rates of protein turnover between animals, there is considerable variation in efficiency of protein gain (Herd et al., 2004). It has been suggested that more efficient cattle experience less protein turnover than inefficient

cattle, therefore requiring less energy for protein deposition. Due to this inter-animal variation in protein turnover, it has been hypothesised that biological differences in protein deposition and fat accumulation may contribute to observed variation in FE (Fitzsimons et al., 2017). However, there are contrasting findings to support this hypothesis. For example, upon conducting a meta-analysis of published literature reporting on growing cattle, Kenny et al. (2018) did not observe any differences in muscle gain or back fat measurements between RFI divergent cattle. This is not in agreement with Berry and Crowley (2013), who noted that in growing cattle, low-RFI animals were more likely to be leaner than high-RFI animals, and inefficient cattle were more likely to have greater body fat measurements than their efficient counterparts. Contrasting findings have also been reported in mature cattle (i.e. those who are no longer growing). Basarab et al. (2007) observed that dams with greater back fat thickness produced low-RFI offspring, while in an Australian study it has been observed that high-RFI cows demonstrated greater levels of fatness than low-RFI cows (Arthur et al., 2005).

1.4.7. The impact of body maintenance on RFI

The proportion of consumed energy required for body maintenance in adult cattle is greater than 50%. Consequently, maintenance is the most important factor in determining efficiency (Caton et al., 2000, Arango and Van Vleck, 2002). The energetic cost of maintaining homeostasis is contributed to by several physiological processes.

1.4.7.1. Energy consumption of the gastrointestinal tract and liver

The gastrointestinal tract (GIT) and the liver are two major energy consumers within the ruminant. Collectively these two organs account for approximately 9.5% of body weight while consuming greater than 40% of energy generated (Fitzsimons et al., 2017). Contrastingly, skeletal muscle comprises greater than 40% of body weight while accounting for approximately 21% of energy use (Caton et al., 2000), further highlighting the extreme energy expenditure of the GIT and liver compared to their weight. The energy demands of these organs has led to differences in their

metabolism or morphology to be suggested as sources of inter-animal variation in energy requirements (McBride and Kelly, 1990, Johnson et al., 1990).

Studies investigating the impact of GIT and liver size have reported contrasting findings. For example, Fitzsimons et al. (2014a) reported that low-RFI bulls had lighter stomach complexes than their inefficient counterparts. This was in agreement with Basarab et al. (2003) who reported that low-RFI steers exhibit lower weight stomach complexes and liver. Additionally, Keogh et al. (2015) observed that bulls undergoing compensatory growth, a form of induced FE, had smaller intestinal complexes than their counterparts which did not experience compensatory growth. Similarly, Zhang et al. (2017) who found that low-RFI lambs had smaller liver and rumen sizes than high-RFI lambs. Contrastingly, several studies have not found any differences in weight of visceral organ weight in RFI-divergent cattle (Cruz et al., 2010, Mader et al., 2009).

1.4.7.2. Maintenance and metabolism of skeletal muscle

Skeletal muscle accounts for approximately 40% of body weight, and is a major contributor to basal energy expenditure via protein turnover and degradation (Caton et al., 2000, Henriksson, 1990). It has been suggested that inefficient cattle exhibited increased rates of muscle degradation, which consequently may lead to increased energy expenditure to maintain muscle mass (Herd and Arthur, 2009). This hypothesis has been supported by others who have reported that RFI is negatively associated with circulating markers of protein catabolism (Fitzsimons et al., 2013). Lines et al. (2014) and Gomes et al. (2013) observed no variation in protein metabolism between RFI-divergent heifers and steers, respectively.

1.4.8. The role of lipid metabolism in feed efficiency

Lipid metabolism refers to the synthesis and degradation of lipids (Nafikov and Beitz, 2007). Lipid metabolism begins upon intake of dietary foodstuffs. Consumed lipids are processed within the rumen and absorbed within the intestine. Dietary fibre

is converted to short chain fatty acids, such as VFAs, by microbes within the rumen and then absorbed across the GIT wall (Nafikov and Beitz, 2007, Janssen, 2010). Following absorption, fatty acids are then metabolised further in target tissues such as the liver and skeletal muscle (Hocquette and Bauchart, 1999). If an animal is consuming excess energy, this may be converted to lipid and deposited as fat which is primarily stored in the adipose tissue (Lawrence et al., 2012b). As previously discussed, lipid deposition and the level of carcass fatness has been suggested to play a role in divergent RFI (Kenny et al., 2018, Berry and Crowley, 2013).

1.4.9. Mitochondria and energetic efficiency

Mitochondria are membrane bound organelles that produce approximately 90% of cellular energy in the form of adenosine triphosphate (ATP) (Nelson et al., 2008). ATP is produced by oxidative phosphorylation in the inner mitochondrial membrane via the transfer of electrons through the electron transport chain (Nelson et al., 2008). The electron transport chain is also a major site of reactive oxygen species (ROS) production (Liu et al., 2002). Normal physiological production of ROS is required for cellular signalling, however, excess production of ROS can be pathogenic (Sena and Chandel, 2012).

Due to the central role of mitochondria in energy production, it has been suggested that variation in mitochondrial function may contribute to divergence in FE (Bottje et al., 2002). Following on from this work, Bottje and Carstens (2009) observed that inefficient animals produce less ATP within their mitochondria due to proton leakage from the inner mitochondrial membrane. Furthermore, it has been illustrated that mitochondrial respiration rate is higher in low-RFI cattle (Kolath et al., 2006, Kelly et al., 2011) and that the production of ROS is less in efficient animals (Bottje and Carstens, 2009).

1.4.10. Blood metabolites and feed efficiency

Blood metabolites and hormones have been considered as physiological markers for RFI and other measures of FE in beef cattle (Kelly et al., 2010a, Wood et al., 2004). For example, serum insulin-like growth factor-I (IGF-I) has been suggested as a

potential selection marker for low-RFI cattle (Wood et al., 2004). However, further work investigating the effect of selection for IGF-I on RFI status found that IGF-I status had minimal effect on RFI (Lancaster et al., 2008). Similarly, several other studies found no relationship between RFI status and IGF-I (Kelly et al., 2010a, Kelly et al., 2010b, Lawrence et al., 2011). However, Welch et al. (2013) suggested that the growth hormone-IGF axis is likely to play a role in RFI-divergence at a molecular level.

Glucose has also been suggested as a potential metabolite marker of RFI (Russel and Wright, 1983). Levels of plasma glucose have been shown to have a contrasting relationship with RFI status. Several studies have demonstrated no relationship between glucose and RFI (Kelly et al., 2010b, Fitzsimons et al., 2014a) however, Kolath and colleagues reported a positive relationship between circulating glucose levels and RFI (Kolath et al., 2006).

Non-esterified fatty acids (NEFA) and beta-hydroxy butyrate (βHB) are ketone bodies that are produced by ketogenesis in the liver. When ruminants are fed below their maintenance requirements, the production of these ketone bodies increases (Hocquette et al., 1998). Published studies report conflicting findings with regards to the relationship between NEFA and RFI. For example, several studies did not observe any relationship between NEFA levels and RFI status (Lawrence et al., 2011, Fitzsimons et al., 2014a) while other studies have found that increased circulating NEFA levels were associated with lower RFI (Kelly et al., 2010a). Similarly, there is little agreement within the literature with regards to the relationship between βHB and RFI. Kelly et al. (2010a) observed a positive relationship between βHB and RFI, while Lawrence et al. (2012a) observed no relationship between RFI and βHB.

1.4.11. Stress physiology and feed efficiency

When animals are stressed, their metabolic rate and energy consumption are increased, via activation of the hypothalamic-pituitary-adrenal (HPA) axis (Knott et al., 2010). Animals under stress divert nutrients away from growth and towards the

stress response (Knott et al., 2008). It has been suggested that variation in the stress response may account for some of the observed variation in RFI (Herd and Arthur, 2009, Welch et al., 2012). Previous results from an adrenocorticotropic hormone (ACTH) challenge on RFI-divergent beef heifers found that low-RFI heifers displayed a reduced sensitivity to ACTH, suggesting that low- and high-RFI animals display differential HPA axis function (Kelly et al., 2017). However, previous work by Kelly et al. (2016) observed no differences in the responsiveness of the HPA axis of RFI-divergent cattle to an exogenous challenge.

1.5. The genome and genomics

The genome refers to all of the genetic information possessed by an organism. DNA, which contains coding and non-coding sequences, is arranged into chromosomes and all genetic information contained within these sequences constitutes the genome (Alberts et al., 2013). Genomics refers to the study of the genome and can be carried out at a DNA, RNA or protein level by employing molecular biology techniques to elucidate a complete dataset of all genes in an organism, and the interactions of these genes with each other and the environment (Alberts et al., 2013). Genomics can be broadly divided into structural and functional genomics (Hocquette et al., 2007). Structural genomics refers to the study of the physical nature of the genome, while functional genomics refers to the investigation of DNA products in a system-wide manner as opposed to studying single genes or proteins (Hieter and Boguski, 1997).

1.5.1. The Bovine Genome Project

The bovine genome was published in 2009 by the Bovine Genome Sequencing and Analysis Consortium (Elsik et al., 2009). The bovine genome sequence, based on DNA taken from the Hereford cow L1 Dominette, contains approximately 22,000 genes with 14,345 of these shared across seven mammalian species (Burt, 2009).

Two *Bos taurus* genome assemblies have been made available based on the sequence data generated from the Bovine Genome Project. The Btau assembly generated by

the Baylor College of Medicine, currently on version 4.6.1, combined whole genome shotgun assembly and bacterial artificial chromosomes to assemble the Btau genome (Liu et al., 2009). Simultaneously, the University of Maryland generated an alternative assembly known as the UMD assembly, this is currently on version 3.1 (Zimin et al., 2009).

Sequencing of the bovine genome provided an important resource to understand the impact of genetic variation in cattle and to accelerate genetic improvement within the beef and dairy industries (Berry et al., 2010).

1.5.2. Variation in the bovine genome

Single nucleotide polymorphisms (SNPs) are single base changes to DNA, found at a particular location within the genome. SNPs, the most common form of genetic variant, and are located in both coding and non-coding regions of DNA (Brookes, 1999). The identification of DNA sequence variants associated with traits of interest is of importance to breeding programmes and facilitates genetic improvement (Goddard and Hayes, 2009). The Bovine HapMap project and the Bovine Genome Project facilitated the identification of SNPs across the bovine genome (Gibbs et al., 2009). Consequently, this has enabled development of genotyping chips, such as the Illumina BovineSNP50 genotyping chip (Matukumalli et al., 2009).

1.5.3. Genome-wide association studies

Genome-wide association studies (GWAS) aim to identify genetic variants that are associated with phenotypic variation in a population (Bush and Moore, 2012). The phenotypic variation under investigation may be disease status, which would be a case-control in nature (i.e. affected or unaffected), or may be quantitative, such as FE (Visscher et al., 2017). Due to the development of genotyping chips and the decreasing costs of genotyping, GWAS have become very popular methods by which to investigate the genetic basis of various phenotypes (Hayes and Goddard, 2010, Visscher et al., 2017).

Most GWAS use SNPs as the genetic variants for which associations are generated. However, the SNPs genotyped within a GWAS may not be responsible for the variation in the phenotype. Linkage disequilibrium (LD) between a genotyped SNP and a causal variant may enable identification of an association signal, even if the causal SNP is not genotyped (Visscher et al., 2017, Balding, 2006). LD is the non-random association between two or more SNPs when they are inherited together (Slatkin, 2008). Consequently, GWAS have utility in identifying variants associated with observed differences in the trait of interest (Bush and Moore, 2012, McCarthy et al., 2008). However, complex traits are typically driven by many genetic variants each with a small effect. Therefore, to identify these variants or variants in LD with the causal SNP a large sample size and a large number of genotyped SNPs are required (Balding, 2006). Recently, the development of high density bovine genotyping chips have facilitated GWAS in cattle (Rincon et al., 2011, Matukumalli et al., 2009).

1.5.3.1. GWAS for feed efficiency

Genetic markers identified via GWAS can be incorporated into genomic assisted selection programmes in order to facilitate breeding of more desirable animals (Hayes et al., 2013). FE is an important economic trait which is difficult and costly to measure at the individual animal level. Consequently, there has been much interest in using GWAS to identify potential genomic biomarkers for RFI to be included in genomic assisted breeding programmes (Table 1.2).

Several association studies have identified SNPs within or nearby to genes that are involved in physiological functions hypothesized to play a role in RFI divergence. For example, several studies have identified SNPs in genes related to metabolism and immune function as associated with RFI (Seabury et al., 2017, Serão et al., 2013b, Abo-Ismail et al., 2014). Similarly, with regards to protein synthesis and degradation, both Karisa et al. (2013) and Abo-Ismail et al. (2018) identified SNPs in *CAST*, a gene which produces a protein which inhibits protein degradation, to be associated

with decreased RFI. While Barendse et al. (2007) and Abo-Ismail et al. (2018) identified variants within *GHR* to be negatively associated with RFI. Cattle QTLdb is a repository which contains a complete list of SNPs and quantitative trait loci (QTL) associated with RFI, and other traits (Hu et al., 2013).

Table 1.2. A selection of association studies carried out to identify genetic variants associated with RFI in beef cattle.

Study	SNP number or chip used	Breed of cattle employed	Sample size	Number of SNPs/QTLs identified
Barendse et al. (2007)	9,260 SNPs	Angus, Brahman, Belmont Red, Hereford, Murray Grey, Santa Gertrudis and Shorthorn	1472 steers	161 SNPs
Nkrumah et al. (2007)	100 microsatellites and 355 SNPs	Crossbred steers sired by Angus, Charolais or Alberta Hybrid bulls	400 steers	8 QTLs
Sherman et al. (2010)	2,633 SNPs	Crossbred steers sired by Angus, Charolais or Alberta Hybrid bulls	464 steers	150 SNPs
Bolormaa et al. (2011)	Illumina 50K and Affymetrix 10K	Three populations. One steer population of purebred Angus, Murray Grey, Shorthorn, Hereford, Brahman, Santa Gertrudis and Belmont Red. One cow population of Brahman and Tropical Composites. A final population of Angus cattle genotyped on the 10K chip	Steers: 852 Cows: 1456 Angus steers genotyped on the10K chip: 379	186 SNPs identified in total across all populations.
Mujibi et al. (2011)	Illumina Bovine 50K SNP chip	Crossbred steers sired by Angus, Charolais or Alberta composite bulls.	728 steers. Two analyses were carried out to minimise relatedness within samples	100 SNPs with a *P*-value less than 0.001 for analysis one. 100 SNPs with a *P*-value less than 0.002 for analysis two.

Study	SNP number or chip used	Breed of cattle employed	Sample size	Number of SNPs/QTLs identified
Snelling et al. (2011)	Illumina Bovine 50K SNP chip	Crossbred steers sired by Angus, Charolais, Gelbvieh, Hereford, Limousin, Red Angus and Simmental bulls.	1,159 steers	15 SNPs associated with RFI with a *P*-value less than 0.0001.
Rolf et al. (2012)	Illumina Bovine 50K SNP chip	Angus steers	698 steers	281 SNPs
Serão et al. (2013b)	Illumina Bovine 50K SNP chip	Angus and Simmental steers	1,321 steers	26 SNPs
Lu et al. (2013a)	Illumina Bovine 50K SNP chip	19 purebred Angus, 3 Charolais, 11 Piedmontese and 718 crossbred cattle. Crossbreds were combinations of Angus, Limousin, Gelbvieh, Piedmontese, Simmental and Charolais	717 males and 34 heifers	0 SNPs
Karisa et al. (2013)	117 SNPs identified within candidate genes	Steers sired by Angus, Charolais or Alberta composite bulls	531 steers	25 SNPs
de Oliveira et al. (2014)	Illumina BovineHD BeadChip	Nellore steers	591 steers	4 QTLs

Study	SNP number or chip used	Breed of cattle employed	Sample size	Number of SNPs/QTLs identified
Abo-Ismail et al. (2014)	339 candidate SNPs identified via a candidate gene approach	726 crossbred beef cattle with an average breed composition of 45.9% Angus, 20.7% Simmental, 5% Piedmontese, 4.2% Gelbvieh, 2% Charolais and 1.4% Limousin	Heifers: 38 Steers: 387 Bulls: 301	11 SNPs
Santana et al. (2014)	Illumina Bovine HD BeadChip and Illumina Bovine 50K SNP chip	Nellore bulls and steers	720 bulls and steers	2 SNPs
Saatchi et al. (2014a)	Illumina Bovine HD BeadChip and Illumina Bovine 50K SNP chip	1,160 cycle VII steers*, 1,658 Angus steers, 840 Hereford steers, 30 Hereford heifers, 1,445 Simmental-Angus cross steers.	5,133 cattle total	10 QTLs
Seabury et al. (2017)	Illumina Bovine HD BeadChip and Illumina Bovine 50K SNP chip	706 Angus steers, 826 Hereford steers, 24 Hereford heifers, 1465 Simmental-Angus crossbred steers	3,021 cattle total	17 QTL
Abo-Ismail et al. (2018)	250 candidate SNPs	284 Hereford bulls, 25 Hereford heifers, 89 Angus heifers, 199 Angus-Hereford crossbred heifers, 23 Hereford steers, 71 Angus steers, 183 Angus-Hereford steers	874 cattle total	63 SNPs

*The breed composition of the cycle VII steers is as described in Snelling et al. (2011). SNPs: single nucleotide polymorphisms; QTL: quantitative trait locus.

1.5.3.2. Uncovering selection markers applicable to multiple breeds

As discussed previously, there is breed heterogeneity in beef production systems in Ireland (Wickham et al., 2012). This represents a difficulty in identifying genetic variants associated with RFI. For genetic markers to be included in the Irish genomic selection breeding programme they must be applicable across multiple breeds and their crosses. This is complicated by the variation in LD between markers of different breeds of cattle (de Roos et al., 2008). Due to variation in LD across breeds it is possible that a SNP associated with RFI in one breed, may not be associated with the trait in another breed or it may be associated with RFI in the opposite direction. A potential method of identifying markers that may be included in a multi-breed genomic assisted selection programme is by using meta-analysis.

Meta-analysis is a method applied to combine summary statistics from individual GWAS in order to generate an across study result for all variants. Meta-analyses have the advantage of enabling a combination of results from several GWAS thus resulting in increased power to detect variants (Evangelou and Ioannidis, 2013). Additionally, meta-analyses reduce the likelihood of false positive associations, as it is unlikely that a false positive association will be present in all breeds analysed, and consequently any potential false positive signals would no longer be significant following meta-analysis (Teissier et al., 2018).

One tool used to carry out meta-analysis is METAL (Willer et al., 2010). METAL combines summary stats from multiple association studies to generate a signed *Z*-score. A very negative *Z*-score indicates that the allele under test is strongly associated with lower trait levels, while a very positive *Z*-score indicates that the allele is associated with increased trait levels (Willer et al., 2010).

As meta-analysis offers the opportunity to combine GWAS results, it has been used to identify genetic markers associated with traits of interest in multiple breeds. For example calving performance, milk production, stature and fat and protein

percentage in milk have all been investigated in multiple populations of cattle using meta-analysis (Purfield et al., 2015, Teissier et al., 2018, Bouwman et al., 2018, Pausch et al., 2017)

1.5.4. Next-generation sequencing

Next-generation sequencing (NGS) refers to high throughput DNA sequencing (Schuster, 2007). The development of this technology has enabled researchers to rapidly profile the entirety of an organism's genomic information (Bai et al., 2012). Available sequencing technologies have included Illumina sequencing, Roche 454 sequencing, Ion Torrent and SoLiD sequencing. NGS approaches result in the production of short sequences, known as reads, which can be interrogated to uncover differences in the DNA-sequence or the gene expression profile of the animal under investigation (Bai et al., 2012). Currently, the Illumina platform is the most commonly used NGS system, accounting for more than 70% of the NGS market (Thayer, 2014).

The Illumina sequencing technology uses a technique known as sequencing-by-synthesis (SBS) to generate reads (Figure 1.4). Initially, the generated library, with adaptors attached to each fragment, is loaded onto a flow cell, and these single stranded DNA molecules bind to oligo-nucleotides on the surface. Once bound, these fragments are PCR amplified to form clusters of identical templates on the flow cell. DNA polymerase then generates a complementary DNA strand from these templates using fluorescently labelled nucleotides. During the incorporation of each nucleotide into the template strand, the attached fluorophore is excited, enabling identification of the newly added DNA base. This occurs over multiple cycles allowing the generation of reads, typically 50 to 100 base pairs in length (Buermans and den Dunnen, 2014).

There are several applications of NGS including whole genome sequencing (WGS), targeted sequencing and sequencing of the entire transcriptome, which is known as

RNA-Seq and is further described in Section 1.5.5.2. WGS refers to sequencing the entirety of an organism's genome, i.e. all coding and non-coding regions. While targeted sequencing enables NGS of only select, pre-determined regions of the genome, often the exome (Bai et al., 2012). Targeted sequencing overcomes the high cost associated with WGS. However, the price of WGS is continually decreasing (Weymann et al., 2017). It is likely that WGS will become a more commonly used tool for SNP discovery (Kumar et al., 2012).

Figure 1.4. Overview of Illumina Sequencing by Synthesis. Initially the fragmented library with attached adaptors attach to the flow cell. The bound fragments then bridge to primers and are PCR amplified. Each bound fragment generates clusters of identical fragments. Sequencing then progresses. During a single cycle, a labelled nucleotide is attached to each template, laser excitation of the newly added fluorophore occurs and an optic scanner collects the signal from each cluster. Once this is complete the next cycle of synthesis may continue (Lu et al., 2016).

1.5.5. Bioinformatics

Bioinformatics can be described as the usage of computer programmes to process, manage and analyse biological data (Altman, 2012). Bioinformatics enables analysis of the large amount of data as generated by next-generation sequencing technologies (Berry et al., 2010). Bioinformatic pipelines enable quality control analysis of generated reads, alignment of the generated reads to a reference genome and further analysis. Further analysis of RNA-Seq generated reads, for example, could include quantification of reads and identification of differentially expressed genes (DEGs) (Conesa et al., 2016).

The first step in bioinformatic analysis of WGS data is often quality control (QC). The first step in QC is trimming of the adaptor sequences using cutadapt (Martin, 2011) and removal of low quality sequence data by FastQC (Andrews, 2010). Following QC, reads are aligned to the bovine reference genome, which can be carried out using STAR aligner, for example (Dobin et al., 2013). STAR also enables quantification of the mapped reads at a gene level. Upon generation of the raw read counts, differential gene expression analysis can be carried out if the transcriptome was sequenced, for example. DESeq2 is a software which can be used to identify DEGs from raw read counts generated by RNA-Seq (Love et al., 2014).

1.5.6. Transcriptomics

Transcriptomics refers to the study of all DNA being transcribed at a given time, which is all RNA transcripts including mRNA, amongst others (Wang et al., 2009). Transcription is the synthesis of RNA, using the DNA template (Figure 1.5). Briefly, pre-mRNA is transcribed from DNA. Within this pre-mRNA, which is complementary to the DNA template, are introns and exons. Pre-mRNA is spliced whereby introns are removed and the exons are combined, forming mature mRNA. Following transcription, the mRNA can be exported from the nucleus and translated into a polypeptide (Alberts, 2015).

The transcriptome of an organism is continually changing in response to the environment. Consequently, transcriptomics offers the opportunity to investigate the effects of variation in the environment on the organism (Wang et al., 2009). The transcriptome can be interrogated using techniques such as microarrays or RNA-Seq and DEGs can be identified.

Figure 1.5. Overview of DNA processing in an eukaryotic cell. First, both coding and non-coding regions of DNA are transcribed into mRNA. Some regions, known as introns, are removed in during initial mRNA processing. The remaining exons are spliced together and the spliced mRNA molecule is prepared for export from the nucleus through the addition of an endcap and a poly-A tail. Once in the cytoplasm, the mRNA can be translated into a protein. Sourced from: https://www.nature.com/scitable/topicpage/gene-expression-14121669.

1.5.6.1. Microarrays

A microarray is a glass-chip based array that contains thousands of specific DNA sequences, known as probes, at predefined positions (Karakach et al., 2010). Microarrays have been used to interrogate the transcriptome of cattle in order to identify differentially expressed genes associated with various traits including FE. Chen et al. (2011) used microarray analysis to identify 161 genes associated with FE. These same authors carried out pathway analysis of their findings, observing that the DEGs identified to be implicated in biological processes such as lipid metabolism and cellular growth and proliferation (Chen et al., 2011).

While microarray analysis provide insight into the biology underpinning variation in RFI, there are several drawbacks associated with this technology. For example, due to microarray's reliance on hybridization it is necessary to have prior knowledge of the sequences being investigated, therefore, novel transcripts cannot be identified (Shendure and Ji, 2008).

1.5.6.2. RNA-Seq

RNA-Seq, a next-generation sequencing technology, provides an alternative to microarrays for transcriptomic profiling. To carry out RNA-Seq, mRNA is fragmented and converted to cDNA, adaptors are ligated to the generated cDNA and these cDNA fragments are then sequenced using high-throughput methodologies as described in Section 1.5.4 (Figure 1.6) (Wang et al., 2009). Following sequencing, reads are aligned to a reference genome and the level of expression for each gene can be calculated and variation in transcription can be investigated (Mortazavi et al., 2008). RNA-Seq can be used to compare transcriptomic profiles of phenotypically divergent cattle. This enables insight into the gene-level differences between groups of cattle (Suravajhala et al., 2016). A primary advantage of RNA-Seq over microarray analysis is that RNA-Seq does not require prior probe selection and can enable discovery of novel genes and transcriptional products, such as splice variants (Wang et al., 2009, Haas and Zody, 2010).

Figure 1.6. Overview of RNA-Seq methodology. Long mRNAs are converted into a library of cDNA fragments. Sequencing adaptors are subsequently added to each cDNA fragment and short sequence reads are obtained from each cDNA using NGS methodology. These are aligned to the reference genome and mapped sequencing reads can be quantified. Adapted from Wang et al. (2009).

1.5.6.3. RNA-Seq of RFI-divergent cattle

RNA-Seq has been widely adopted to investigate the transcriptome of cattle with divergent RFI profiles and identify DEGs associated with this trait. Table 1.3 lists a selection of these studies which employed this technique. The identification of DEGs can further our understanding of the biology controlling RFI variation by enabling investigation into the biological roles of these genes. RNA-Seq studies have identified pathways such as lipid metabolism, mitochondrial function and nutrient uptake as being implicated in RFI-divergence (Mukiibi et al., 2018, Tizioto et al., 2015, Tizioto et al., 2016, Kong et al., 2016b).

Lipid metabolism and fat deposition are both energetically costly physiological processes. Therefore, it is not surprising that variation in the expression of genes implicated in lipid metabolism have been associated with divergence in FE by several studies (Mukiibi et al., 2018, Salleh et al., 2017). A recent study examining hepatic gene-expression of three breeds of Canadian cattle reported downregulation of hepatic lipid metabolism in low-RFI steers (Mukiibi et al., 2018). While functional enrichment analysis of genes differentially expressed in the hepatic transcriptome of dairy cows divergent in RFI also revealed over-abundance of genes related to lipid metabolism (Salleh et al., 2017). Similar results were observed in the skeletal muscle transcriptome of Nellore steers whereby DEGs were noted to be involved in processes such as lipid metabolism and lipid transport (Tizioto et al., 2016).

Using transcriptomic methodologies, genes and pathways associated with mitochondrial function have been identified as differentially expressed between high- and low-RFI animals. For example, is was observed that low-RFI cattle displayed greater expression of mitochondria-related genes in the rumen epithelium (Kong et al., 2016b). Similar findings have been reported in skeletal muscle (Tizioto et al., 2016) and liver (Tizioto et al., 2015) of RFI-divergent cattle. These results potentially indicate that feed efficient cattle can produce energy more efficiently than feed-inefficient cattle.

Several studies have also noted differences in expression of genes related to oxidative stress. For example, it has been observed that *GSTM1* was differentially expressed in RFI divergent cattle. Chen et al. (2011), using microarray analysis, found that *GSTM1* levels were decreased in low-RFI Angus Bulls. In contrast to this Tizioto et al. (2015) and Paradis et al. (2015) observed that *GSTM1* levels were increased in feed efficient cattle.

With regards to nutrient absorption, Kong et al. (2016b) hypothesised that low-RFI cattle would be more efficient in VFA metabolism and expression of genes

implicated in this process would differ between high- and low-RFI cattle. However, upon transcriptomic analysis of the rumen epithelium no difference in such genes was observed (Kong et al., 2016b). However, these same authors observed differential expression of genes implicated in cytoskeletal organisation, cell adhesion and cell migration. They hypothesised that low-RFI cattle may absorb nutrients at a higher rate, enabling greater energy uptake (Kong et al., 2016b).

Table 1.3. A selection of RNA-Seq studies to identify DEGs associated with divergent RFI in beef cattle.

Study	Sample size and breed	Tissue investigated	Number of DEGs identified
Paradis et al. (2015)	Ten high and ten low RFI heifers selected for RNA-Seq. Within each group there were seven Hereford-Aberdeen Angus crossbreds and three Charolais-Red Angus-Main Anjou crossbreds.	Liver	7
Tizioto et al. (2015)	Ten high RFI and ten low RFI Nellore steers.	Liver	112
Alexandre et al. (2015)	Twenty high and twenty low RFI Nellore steers/	Liver	8
Tizioto et al. (2016)	Ten high RFI and ten low RFI Nellore steers.	Skeletal muscle	73
Weber et al. (2016)	Eight high RFI and eight low RFI Angus steers.	Pituitary gland, skeletal muscle, liver, adipose tissue, duodenum.	Pituitary gland: 285 Skeletal muscle: 220 Liver: 275 Adipose tissue: 33 Duodenum: 213
Kong et al. (2016b)	Nine high RFI and nine low RFI Hereford-Angus crossbred steers	Rumen epithelium	122
Salleh et al. (2017)	Five and four high and low RFI Holstein cows, respectively, and five low and five high RFI Jersey cows.	Liver	Holstein: 70 Jersey: 19
Khansefid et al. (2017)	Twenty-two high RFI and twenty-one low RFI Angus bulls for skeletal muscle transcriptomics. Twenty-three and fourteen Angus bulls used for liver transcriptomic analysis. Nine high RFI and ten low RFI Holstein cows for liver and blood transcriptomic analysis.	Liver, skeletal muscle and blood.	Angus skeletal muscle: 922. Angus liver: 768. Holstein liver: 473 Holstein blood: 4,817

Study	Sample size and breed	Tissue investigated	Number of DEGs identified
Mukiibi et al. (2018)	Six high RFI and six low RFI Angus, Charolais and Kinsella Composite steers.	Liver	Angus: 72 Charolais: 41 Kinsella Composite: 175 5 DEGs were common to all breeds

RFI: Residual feed inake; DEG: Differentially expressed genes.

1.5.6.4. How differentially expressed genes can aid in genomic selection

The identification of DEGs can also aid in informing breeding programmes via targeted resequencing. Targeted resequencing involves identifying genomic regions of interest, capturing the regions of interest, for example DEGs, and sequencing only those regions (Gnirke et al., 2009, Hodges et al., 2007). These regions of interest are then interrogated for SNPs which may be used for genomic selection (Mullen et al., 2012). Targeted resequencing has been used to identify SNPs within genes of the somatotropic axis causal for variation in calving interval (Mullen et al., 2012). With respect to FE, Cohen-Zinder et al. (2016) used targeted sequencing to identify one SNP associated with variation in RFI across three dietary stages and ages for Holstein calves. Those authors carried out a literature review to identify potential genes of interest for which custom probes were created to capture the regions to be sequenced. The primary advantage of targeted resequencing when compared to WGS is the lower associated cost (Xuan et al., 2013). However, as the expense associated with WGS continues to decrease, it is possible that targeted resequencing for SNP discovery will be replaced by WGS (Green et al., 2011).

1.5.7. Expression quantitative trait loci analysis for genomic selection

As previously discussed, GWAS do not necessarily identify the genetic variant associated with variation in the trait (Visscher et al., 2017). In order to include variants within a genomic assisted breeding programme, markers which explain a proportion of genetic variance must be identified. One method by which causal variants can be identified is by carrying out expression quantitative loci (eQTL) analysis. eQTL analysis is carried out by generating associations between genotypic data with gene expression levels. Consequently, an eQTL is a genetic locus that affects the transcription levels of a specific gene (Figure 1.7, Gilad et al. (2008)). There are two types of eQTL, *cis*- and *trans*-eQTLs. *Cis*-eQTLs are located nearby to the gene which encodes the transcript under investigation while *trans*-eQTLs are located distally to the gene (Cheung et al., 2005). *Cis*-eQTLs have been demonstrated to account for a greater proportion of genetic variation than *trans*-eQTLs (Hughes et al., 2006).

While the identification of eQTLs represents a method to identify genetic loci that contribute to variation in traits of interest, this analysis has not been widely used in livestock populations. González-Prendes et al. (2017) carried out eQTL analysis to uncover variants associated with pork quality traits. Littlejohn et al. (2016) used eQTL analysis to identify genes as causative in the development of divergent milk production traits while Brand et al. (2016) performed eQTL analysis to identify variants associated with temperament. However, in terms of RFI, few such studies have been carried out. Recently, eQTL analysis was carried to aid in understanding of the biological pathways underlying FE in dairy cattle (Salleh et al., 2018). Those authors observed that lipid metabolism and immune function appeared to be the pathways driving RFI variation in Holstein and Jersey cows, respectively. To date, no eQTL analysis has been performed for FE-related traits in beef cattle.

Figure 1.7. An example of the genotype-gene expression relationship that may be observed as an eQTL. This example illustrates that the presence of the G allele has an effect on gene expression. The G allele increases gene expression of this target gene, and if this effect was found to be statistically significant it could be concluded that an eQTL is present. Adapted from Nica and Dermitzakis (2013).

1.5.8. Network Biology

Genes, and their products, interact with each other in a system-wide manner, rather than in distinct linear pathways, to contribute to the observed phenotype (Carter et al., 2013, Charitou et al., 2016). Gene expression data as generated by transcriptomic analyses can be used to carry out co-expression network analysis of genes (Kogelman et al., 2014). This approach generates networks of genes which have similar expression levels, and enables analysis of these constructed networks (van

Dam et al., 2018). Identification of key genes, also known as hub genes, may uncover genes that are integral to the biological control of complex traits (Alexandre et al., 2015). Hub genes are genes that are highly connected within a network and therefore, are more likely to be integral to the system (Zotenko et al., 2008). It has also been suggested that variants within hub genes may have use as potential biomarkers due to the role of hub genes in biological pathways associated with the trait of interest (Kadarmideen et al., 2011). Identification of key genetic biomarkers for FE would enable their inclusion in genomic assisted breeding programmes allowing selection of feed efficient animals.

A common method of carrying out co-expression network analysis is by using weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008). WGCNA clusters genes into modules, which are collections of highly connected genes (Langfelder and Horvath, 2008). To create modules, WGCNA first calculates the similarity between gene expression profiles using Pearson's correlation and then calculates the topological overlap measure (TOM), which represents the interconnectivity of genes. Based on their interconnectivity, genes are grouped into modules. These modules can then be correlated with phenotypic data using Pearson's correlation. This correlation is known as the eigengene significance (Langfelder and Horvath, 2008, Zhang and Horvath, 2005, Suravajhala et al., 2016).

Network biology approaches have been utilized to investigate the underlying biology of FE in livestock species. Alexandre et al. (2015) identified four gene modules associated with RFI in the liver transcriptome of Nellore steers using WGCNA. Those authors observed that genes within the identified modules were associated with processes such as lipid metabolism, oxidation-reduction processes and the immune response. While Kong et al. (2016b) identified three modules associated with RFI following WGCNA of the rumen epithelium of RFI-divergent steers. Pathways enriched within these modules included protein synthesis and oxidative phosphorylation. Similarly, Ramayo-Caldas et al. (2018) observed four modules associated with FE in the liver and duodenal transcriptome of pigs using WGCNA. Enriched pathways observed within these modules included oxidative stress,

inflammation and lipid metabolism. The findings of these studies indicate that lipid metabolism, the immune response and oxidative phosphorylation may be implicated in RFI-divergence.

1.6. The Irish national cattle selection programme

Selective breeding allows future production to be predicted and enables desirable traits to be passed on to later generations (Golden et al., 2009). Selective breeding should be driven by a breeding objective, which is a collection of traits that are necessary for the success of future production objectives (Garrick, 2011). For beef enterprises, the breeding objective is to enable future generations to be more efficient in the production of beef while not negatively impacting traits such as calving ease and gestation length which also have a direct impact on profits (Amer et al., 2001).

In Ireland, the Irish Cattle Breeding Federation (ICBF) is responsible for driving genetic improvement. The ICBF was formed in 1997 with the goal of making Irish farming more profitable (Wickham et al., 2012) and breeding objectives were established for beef cattle in 2001 (Amer et al., 2001). The ICBF ranks animals in terms of economic breeding indexes (EBI). EBI are multi-trait indexes which focus on selection for both production and health-related traits, first introduced in Ireland for beef and dairy cattle in 2001 (Veerkamp et al., 2002, Amer et al., 2001). The ICBF currently implements two main indexes within the G€N€ IR€LAND programme, which aims to improve the genetic profile of the national suckler herd: a terminal index and a maternal index. The terminal index is related to an animal's genetic ability to produce cattle for slaughter while the maternal index reflects an animal's ability to produce genetically and economically desirable daughters. On the ICBF website, animals are given both a maternal and terminal index in Euro terms which are relative to the performance of a given animal's offspring. That is an animal with a terminal index of €100 is expected to produce offspring that are €100 more profitable at slaughter than an average animal (ICBF, 2013). In order to calculate these EBIs, the ICBF uses a combination of progeny testing and, more recently, genomic selection.

1.6.1. Genomic selection

Genomic selection utilises genetic markers located throughout the genome to estimate breeding values (Goddard and Hayes, 2007). In order to establish markers that can be used for genomic selection, many animals of known phenotype must be genotyped, and links between genetic markers and phenotypes must be established (Meuwissen et al., 2001). Genomic selection relies on the identification of QTL which are genomic locations where genetic variations, often SNPs, are associated with phenotypic variation. QTL are chromosomal regions that have been demonstrated to contribute to a particular quantitative trait, such as FE (Herd and Bishop, 2000, Meuwissen et al., 2001). The effect of each identified QTL has on the trait of interest is summed and allows the calculation of a genomic estimated breeding value (GEBV). Animals may then be ranked by GEBV and animals that have desirable genetics for the trait of interest can be easily identified (Goddard and Hayes, 2007).

A major advantage of genomic selection is the potential identification of genetically desirable animals at an early age. Once GEBVs are calculated, an animal may be genotyped at birth, removing the need for progeny testing (Schefers and Weigel, 2012). The almost immediate calculation of an animal's GEBV leads to a reduction in the generation interval, consequently accelerating the speed of genetic improvement (Goddard and Hayes, 2009).

Another advantage of genomic selection is the potential to be able to select cattle for complex traits, such as FE, rapidly without the need to phenotype all animals. In order to calculate the FE of an animal, expensive and labour intensive feeding trials must be carried out. It is untenable to conduct these at the individual producer level. Therefore, the identification of SNPs associated with FE and subsequent calculation of a GEBV for FE would enable rapid and cost effective identification of desirable cattle without the requirement for continual expensive feeding trials (Fitzsimons et al., 2017).

1.6.1.1. Genomic selection in Ireland

The ICBF administers the genomic selection programme in Ireland. Currently, the ICBF database has more than 1,000,000 genotypes (McClure et al., 2018). Genomic selection in Ireland has been made possible by the design of the International Dairy and Beef (IDB) custom microarray chip (Mullen et al., 2013). The IDB chip was developed in order to provide a genotyping chip for the cattle industry in Ireland. This chip contains SNPs for genomic selection, parentage verification, lethal recessives detection and research SNPs (Mullen et al., 2013). However, this chip does not contain SNPs which have been validated for FE in Irish populations of cattle. As mentioned above, a major hindrance to uncovering SNPs for FE is the difficulty in obtaining sufficient phenotypes for these traits (Fitzsimons et al., 2017). Furthermore, the breed heterogeneity of the beef cattle population in Ireland represents a further challenge in identifying applicable SNPs to the Irish population of beef cattle (Wickham et al., 2012).

1.6.1.2. Genomic selection in beef cattle

Genomic selection represents an attractive method to improve the genetic gain of beef cattle (Hayes et al., 2013). Traits such as FE, meat quality and fertility traits are key drivers of profitability in beef production enterprises. These traits are difficult to measure and would require progeny testing. Therefore, if accurate GEBVs were calculated for these traits, significant improvements in the profitability of beef production would be achieved (Hayes et al., 2013, Fitzsimons et al., 2017). Despite the potential benefits of genomic assisted selection breeding programmes for beef cattle, adoption of these technologies have been slow. The primary issue with adoption of genomic selection for beef cattle is the heterogeneous nature of beef herds internationally. While dairy herds are dominated primarily by Holstein-Friesian cattle, beef herds consist of multiple breeds, as well as crossbred animals (Hayes et al., 2013). This multiplicity of breeds represents a challenge to generate reference populations large enough to calculate accurate GEBVs (Hayes et al., 2013). It has been suggested that multi-breed reference populations may overcome this barrier and enable calculation of accurate GEBVs (Khansefid et al., 2014, Lund et al., 2014).

1.6.1.3. Genomic selection for RFI

Due to the heritability and underlying genetic basis for RFI, it is an ideal candidate for genomic selection (Crews Jr, 2004, Kenny et al., 2018). Moreover, due to the difficulties associated with measuring RFI, genomic selection represents a method to incorporate feed efficient cattle into the breeding herd while reducing the cost of phenotyping large numbers of animals (Fitzsimons et al., 2017). The identification of SNPs related to RFI would enable genomic assisted selection for this trait (Kenny et al., 2018). Furthermore, as RFI is a complex and polygenic trait, it is likely that the identification of causal variants will improve the accuracy of calculated GEBVs for mixed breed and crossbred cattle populations (Snelling et al., 2013). Many physiological functions affect FE and the identification of key genes and pathways implicated in these processes may enable identification of causal genetic variants associated with RFI (Herd and Arthur, 2009).

1.7. Aims and objectives

As outlined in previous sections, considerable work has been carried out in order to identify biomarkers for RFI suitable for inclusion in genomic breeding programmes. However, several gaps in the knowledge exist with regards to discovering biomarkers for RFI suitable to Irish and international beef production enterprises.

To date no GWAS have been carried out to uncover SNPs applicable to an Irish population of beef cattle. Such a GWAS would need to include multiple breeds and crossbred cattle. Furthermore, no eQTL have been identified for RFI in beef cattle. eQTL analysis may aid in prioritising candidate SNPs as potential causal variants for RFI-divergence consequently furthering our understanding of the biology of RFI.

Cattle have been observed to re-rank in terms of RFI dependant on diet, age and breed (Durunna et al., 2012, Coyle et al., 2016, Coyle et al., 2017, Crowley et al., 2010). Consequently, biomarkers must be consistent across all of these variables prior to their inclusion in genomic assisted breeding programmes. RNA-Seq may be

used to identify potential candidate genes for further interrogation to uncover biomarker SNPs. However, RNA-Seq analyses carried out to date have only investigated differential gene expression across single dietary phases, while only one analysis has investigated the commonality of DEGs across breeds (Mukiibi et al., 2018). Therefore, the identification of candidate genes associated with RFI regardless of diet and breed is crucial to enable incorporation of robust biomarkers for RFI into genomic assisted breeding programmes as well as increasing our knowledge of the biology underpinning RFI.

RNA-Seq offers insight into single DEGs which influence RFI, however in biological systems single genes do not work in isolation (van Dam et al., 2018). Systems biology approaches offer the ability to uncover groups of genes and key regulatory genes, known as hub genes, which are associated with traits of interest, such as RFI (Suravajhala et al., 2016). However, to the best of my knowledge, no systems biology approaches have been employed to identify hub genes associated with RFI across multiple dietary stages for multiple breeds of cattle. The identification of hub genes would both identify candidate genes associated with RFI and enable investigation into the biological variation underpinning RFI phenotype.

Therefore, the aims of this thesis were:

(i) To identify genetic variants associated with RFI applicable to Irish beef cattle for inclusion in the Irish genomic assisted breeding programme. To achieve this a GWAS was carried using a mix-breed population of Irish beef cattle. To study the effects of RFI-associated variants on the expression of nearby genes, eQTL analysis was utilized. This study is detailed in Chapter 2.

(ii) To identify DEGs associated with RFI-divergence in two breeds of steers during three dietary phases throughout their lifetime. To meet this aim RNA-Seq analysis of the hepatic tissue of RFI-divergent steers offered three varying diets was conducted. Functional enrichment analysis of

DEGs was conducted to gain insight into the biology of RFI-divergence across dietary phase and breed. This study is detailed in Chapter 3.

(iii) To use systems biology approaches to identify hub genes and modules associated with RFI-divergence in steers offered different diets throughout their lifetime. Hub genes, which may act as key regulators of biological processes pertaining to RFI, will be candidates for further work. Gene set analysis of modules will aid in understanding which biological processes underlie RFI-divergence. This study is detailed in Chapter 4.

Chapter 2

GWAS and eQTL analysis identifies a SNP associated with both residual feed intake and *GFRA2* expression in beef cattle.

Preamble to Chapter 2: Statement of contribution

This chapter consists of work which was carried out as part of a collaborative project between NUI Galway, Teagasc and ICBF. I gathered all phenotypic data for this study in conjunction with Dr. Claire Fitzsimons. I collated all genotypic data analysed and carried out all analyses within this study. I prepared the manuscript with Dr. Sinéad Waters and Dr. Derek Morris. All authors contributed to data and analyses within this work. This study has been published in Scientific Reports (Higgins et al., 2018)

2. GWAS and eQTL analysis identifies a SNP associated with both residual feed intake and *GFRA2* expression in beef cattle.

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2.1. Abstract

Residual feed intake (RFI), a measure of feed efficiency, is an important economic and environmental trait in beef production. Selection of low RFI (feed efficient) cattle could maintain levels of production, while decreasing feed costs and methane emissions. However, RFI is a difficult and expensive trait to measure. Identification of single nucleotide polymorphisms (SNPs) associated with RFI may enable rapid, cost effective genomic selection of feed efficient cattle. Genome-wide association studies (GWAS) were conducted in multiple breeds followed by meta-analysis to identify genetic variants associated with RFI and component traits (average daily gain (ADG) and feed intake (FI)) in Irish beef cattle (n=1492). Expression quantitative trait loci (eQTL) analysis was conducted to identify functional effects of GWAS-identified variants. Twenty-four SNPs were associated ($P<5x10^{-5}$) with RFI, ADG or FI. The variant rs43555985 exhibited strongest association for RFI (P=8.28E-06). An eQTL was identified between this variant and *GFRA2* (P=0.0038) where the allele negatively correlated with RFI was associated with increased *GFRA2* expression in liver. *GFRA2* influences basal metabolic rates, suggesting a mechanism by which genetic variation may contribute to RFI. This study identified SNPs that may be useful both for genomic selection of RFI and for understanding the biology of feed efficiency.

2.2. Introduction

Feed can account for more than 75% of variable costs of beef enterprises (Finneran et al., 2010). Consequently, selection of cattle that efficiently convert feed to carcass growth would improve farm profits due to reducing expenditure on feed while maintaining protein output (Berry and Crowley, 2013). Moreover, there is pressure on the agricultural industry to reduce methane emissions and improve its environmental footprint, while simultaneously increasing beef output to meet the growing demand for protein worldwide (Ripple et al., 2014). Selection for feed efficient cattle could increase beef output while concurrently decreasing methane production, as it has been suggested that low residual feed intake (RFI) (feed efficient) animals emit less methane than their high RFI counterparts (Fitzsimons et al., 2013).

RFI is a measure of feed efficiency, defined as the difference between actual and predicted feed intake (FI) (Koch et al., 1963). RFI has been shown to be moderately heritable, with an estimated heritability of 0.33 (Kenny et al., 2018, Berry and Crowley, 2013), making it an ideal trait for selection as any genetic gain will be maintained and propagated through the cattle herd (Kenny et al., 2018). However, calculation of RFI is currently impeded by both the expense and logistics associated with its measurement, involving recording of both FI and body weight gain for each individual animal up to a period of 70 days (Nielsen et al., 2013). Identification of genetic markers for RFI and component traits, such as FI and average daily gain (ADG), and their incorporation into genomic assisted breeding programmes would enable more rapid and cost effective selection of feed efficient cattle (Cole and VanRaden, 2017). Indeed, RFI has been incorporated into the Australian dairy industry's genomic breeding programme (Pryce et al., 2015). Unlike the situation that predominates for dairy production systems worldwide, effective identification of selection markers of RFI for beef cattle must take into account a multiplicity of breeds and the mainly crossbred nature of cattle typically utilised within the global beef industry through employing multi-breed populations in order to identify variants of interest (Hayes et al., 2013, Rolf et al., 2014). Differences in linkage disequilibrium (LD) between breeds may impact the association of markers and quantitative trait loci across breeds. The use of multiple breeds in a reference population is important to account for this variation in LD between breeds (Hayes et al., 2013).

In Ireland, the genomic assisted breeding programme is administered by the Irish Cattle Breeding Federation (ICBF) (Wickham et al., 2012). Markers for selection are included on the International Dairy and Beef (IDB) custom genotyping chip, which is based on the Illumina BovineSNP50 genotyping chip providing the IDB chip with genome-wide coverage (Mullen et al., 2013, Inc., 2016). As well as single nucleotide polymorphisms (SNPs) that are used for the genomic selection programme, the IDB chip contains SNPs for parentage verification and SNPs included for research purposes only (Mullen et al., 2013). This includes a selected subset of SNPs associated with RFI in cattle populations outside of Ireland (Rolf et al., 2012, Abo-Ismail et al., 2014, Sherman et al., 2008, Nkrumah et al., 2007, Serão et al., 2013b,

Barendse et al., 2007, Alexandre et al., 2014) which have been added to the IDB chip for research purposes to validate their use as biomarkers of RFI and associated traits in Irish beef cattle (n=102, Supplementary Table 2.1).

Genetic markers for RFI can be identified via genome-wide association studies (GWAS). Several GWAS have identified SNPs associated with feed efficiency-related traits in cattle populations, both purebred and crossbred, from North America, South America and Australia (Barendse et al., 2007, Seabury et al., 2017, Weber et al., 2012, Abo-Ismail et al., 2014, Alexandre et al., 2014). Despite considerable interest in identifying markers for RFI, ADG and FI, no published GWAS has been carried out to test for associations between SNPs and these traits in Irish beef cattle. In Ireland, commercial beef cattle are mainly crossbreds, with Charolais (CH), Limousin (LM), Aberdeen Angus (AA), Belgian Blue (BB) and Simmental (SI) breeds predominating genetically (Wickham et al., 2012). This breed heterogeneity coupled with the challenges in obtaining sufficient numbers of RFI phenotypes for GWAS are primary reasons for the difficulty in applying GWAS on a large-scale basis for RFI to beef cattle in Ireland and most other beef producing nations.

It is important to identify genetic variants that underlie phenotypic variation. One method to identify SNPs that are implicated in observed variation is by carrying out expression quantitative trait loci (eQTL) studies. eQTL analysis enables investigation of the effect of genotype on gene-expression levels which may in turn affect phenotype (Nica and Dermitzakis, 2013). Previous eQTL analysis carried out in mammary tissue of dairy cattle has identified several eQTLs for milk production traits enabling the identification of genes such as *PLAG1* and *MGST1* as potentially functional in the development of divergent milk production traits (Fink et al., 2017, Littlejohn et al., 2016). eQTLs have been identified for temperament in the adrenal cortex of crossbred German cattle (Brand et al., 2016). Despite the ability of eQTL analysis to identify potentially causative genes for complex traits, to the best of the authors' knowledge no eQTL analysis has been carried out for RFI in beef cattle, or in any other livestock species. Liver and muscle are key tissues of interest with regards to feed efficiency as they are both large, metabolically active tissues

accounting for approximately 24% and 25% of basal energy expenditure, respectively (Fitzsimons et al., 2014c, Keogh et al., 2016a). Thus, investigation into the presence of eQTLs in these tissues may aid in unravelling the biology underlying divergence in feed efficiency.

Due to multiple breeds of cattle present in beef production systems, it is important to identify markers for traits that have effects across multiple breeds (Hayes et al., 2013). Thus, the objectives of this study were to: (i) perform GWAS for RFI, and its component traits, namely FI and ADG, in different breeds of Irish beef cattle and combine results in order to identify associated SNPs in a mixed breed cohort, (ii) validate a selection of internationally identified markers of RFI present on the IDBv3 chip for utility as selection markers for RFI in Irish cattle and (iii) to investigate the effects of associated variants on gene expression in metabolically important tissues using eQTL analysis, in order to understand the biological mechanisms underlying divergence in RFI and component traits.

2.3. Materials and methods

All biological sampling and procedures involving animals within this study were reviewed by the Teagasc Animal Ethics Committee and/or the UCD Animal Research Ethics Committee. All procedures carried out prior to 2013 were licenced by the Irish Department of Health and all procedures carried out since 2013 were licenced by the Irish 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.

2.3.1. Phenotypic data collation

Data were collated for this study from growing bulls (n = 1823), steers (n = 459) and heifers (n = 164), which had previously undergone phenotypic measurement testing in Ireland between 2006 and 2017. The average ages and standard deviations for cattle included in the phenotypic data file were available on a group-by-group basis (Supplementary Table 2.2). Throughout each phenotypic measurement trial, the

health of the animals was monitored. Any animal that required treatment was noted and excluded from further analysis. Phenotypes were gathered at the national beef research centre in Teagasc Grange; UCD Lyons Research Farm, University College Dublin and the ICBF national beef performance test station, Tully, Co. Kildare, Ireland. Phenotypes were collected from both purebred and crossbred beef cattle. For crossbred animals to be included in the phenotypic dataset the proportion of genetic material from a single parental breed had to be greater than 50%. Prior to further analysis cattle were grouped by breed. Predominant breeds were LM, CH, SI, BB and AA (n = 737, 499, 413, 191 and 174, respectively), other breeds were represented at smaller numbers. The RFI range for LM, CH, SI, BB and AA was 2.69 to -2.52, 2.70 to -2.48, 2.75 to -2.82, 1.63 to -2.00, and 2.87 to -2.64, respectively.

The resulting phenotypic file consisted of 2,446 cattle. For 429 of these animals, data relating to breed, diet, and methods used to calculate RFI, ADG and FI has been previously described (Coyle et al., 2016, Clarke et al., 2009, Fitzsimons et al., 2014c, Keogh et al., 2015, Lawrence et al., 2012a, Fitzsimons et al., 2013). Records for remaining cattle were made available by the ICBF from the national beef performance test centre at Tully, Co. Kildare, Ireland.

The management protocol of the ICBF animals is described briefly here. Animals were housed in pens for the duration of their test period which was between 70 and 105 days. A Calan gate system (American Calan, Northwood, NH, USA) was used to record individual animal FI. Bulls were individually offered *ad libitum* concentrates and 3kg fresh weight of hay, while steers were offered 8kg concentrates and 5kg fresh weight of hay. Hay was offered in order to maintain healthy rumen function and to reflect an Irish commercial high concentrate based dietary regimen. Refused feed was weighed weekly and subtracted from total feed offered in order to calculate total feed consumed. Dry matter intake was then calculated in order to determine FI, which was used for calculation of RFI. Cattle were weighed at the beginning and end of the test period, and every 21 days during the test period. ADG was calculated as the coefficient of linear regression of body weight on time, computed in the software

package R (Team, 2014). Mid-test metabolic bodyweight (body weight$^{0.75}$, MBW) was calculated as body weight$^{0.75}$ 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 through all MBW observations. RFI was calculated for each animal as the difference between actual and predicted FI. Predicted FI for each animal was computed by regressing FI on MBW and ADG. Calculation of predicted FI was calculated for each contemporary group individually.

2.3.2. Genotyping

DNA was isolated for genotyping from one of two tissue types sourced from 429 cattle described previously. Muscle was used when blood was unavailable. Blood samples were obtained by jugular venepuncture at the end of the RFI measurement period, as per Fitzsimons et al. (2013), and stored at -80°C prior to use. Muscle samples were obtained via biopsy of the *M. longissimus dorsi* following the RFI measurement period, as per Kelly et al. (2011), and stored at -80°C before DNA extraction. DNA from blood samples was extracted using the Maxwell 16 Blood DNA kit (Promega, Madison, WI, USA) as per manufacturer's instructions. DNA was extracted from muscle samples using a phenol-chloroform extraction method. Briefly, 0.1g of frozen muscle tissue was immersed in 1mL of Trizol and homogenized using a Precellys 24 homogeniser. 200µl chloroform was added to the homogenate, which was then centrifuged at room temperature for five minutes at 16,000g. After centrifugation the aqueous phase was transferred to a new tube. Two volumes of ice cold ethanol were added to the aqueous phase and this mixture was centrifuged at 16,000g for 15 minutes at 4°C resulting in the formation of a DNA pellet. The supernatant was removed and the pellet was washed by the addition of 1ml 70% ethanol and centrifuged at 16,000g for 5 minutes at 4°C. Washing was carried out twice. Following washing, any remaining supernatant was removed and the pellet was left to air-dry. The DNA was then re-suspended in 150µl RNase/DNase free H_2O.

Once DNA was isolated, samples were analysed for quality and quantity using a Nanodrop spectrophotometer. DNA of sufficient quality for genotyping was

available for 422 samples. All DNA samples were normalised to a concentration of 50ng/µl for genotyping analysis. Genotyping was carried out on the IDBv3 chip (Mullen et al., 2013) by Weatherby's Scientific Ltd. (Johnstown, Naas, Co. Kildare, Ireland). The ICBF provided genotypes for 1,262 cattle that had been genotyped on the IDBv3 chip by Weatherby's Scientific.

In addition to the 1,684 animals genotyped directly on the IDBv3 chip, 338 cattle were genotyped on the Illumina Bovine HD genotyping chip. These 338 cattle were imputed to IDBv3 density using Fimpute version 2.2 (Sargolzaei et al., 2014). The reference population used for Fimpute was 50,000 Irish cattle with genotyped parents. Imputation of all 338 cattle was conducted across breed type to reflect the Irish national cattle population.

Once genotyping and imputation were complete the study consisted of 2,022 animals with genotypic data for all IDBv3 markers. This genetic data were uploaded to the SNP Variation Suite (SVS) environment (Golden Helix, Version 7.7.6).

2.3.3. Preparation of files for analysis

Quality control (QC) was carried out on genotypes imported into the SVS environment. SNPs were removed from analysis if they had a call rate of less than 0.80 or a minor allele frequency of less than 0.05. Cattle were removed from analysis if they had a call rate of less than 0.95. Following QC, 2,008 cattle and 44,338 markers remained for analysis. LD pruning was carried out at r^2 threshold of 0.5 and 7,841 markers were discarded following pruning (Laurie et al., 2010). The remaining 36,496 SNPs that passed all QC measures were acceptable for further analysis (Supplementary Table 2.3).

The collated phenotypic data were merged with the genotype data, creating a dataset containing 1,822 cattle eligible for analysis. A genomic kinship matrix was computed from the population, which was included as a covariate in the GWAS in order to

account for relatedness. From this dataset, cattle from five beef breeds were analysed (n=1492). The breeds included in the analysis were AA, BB, CH, LM and SI (n=102, 177, 387, 537 and 289, respectively).

2.3.4. Genome-wide association studies

GWAS were carried out in the SVS environment of Golden Helix using a mixed linear model method, EMMAX (Kang et al., 2010), for each breed individually. GWAS resulted in the generation of summary statistics for each trait of interest, i.e. RFI, ADG and FI, for each breed (AA, BB, CH, LI, and SI).

2.3.5. Meta-Analysis

Following initial breed specific GWAS, meta-analyses were carried out for each trait using the software package METAL (Willer et al., 2010). METAL combines *P*-value and direction of effect from each GWAS to conduct Z-score method meta-analysis. METAL analysis results in two outputs, the Z-score for each SNP and a *P*-value for each SNP. A large positive Z-score results in a small *P*-value providing evidence that the allele positively associated with the trait under test. Conversely, a large negative Z-score results in a small *P*-value, showing an allele is negatively associated with the trait (Willer et al., 2010). A *P*-value of less than $5x10^{-5}$ was used to denote genome-wide significance as per recent GWAS studies (Seabury et al., 2017).

2.3.6. Validation of internationally identified RFI SNPs in Irish beef cattle

The inclusion of internationally identified RFI SNPs (n=102, Supplementary Table 2.1) in the current study enabled investigation of their role as markers for feed efficiency in an Irish population of beef cattle.

2.3.7. Functional annotation of genes

Functional annotation of candidate genes was carried out to gain insight into the underlying biology of RFI, ADG and FI. Database for Annotation, Visualisation and Integrated Discovery (DAVID, version 6.8; Huang et al. (2007)) was used for

functional annotation. From the meta-analysis, a list of candidate genes was generated using Ensembl's Variant Effect Predictor. The list contained the nearest gene within a 500kb window to each nominally significant SNP (P<0.05). DAVID assigned genes to pathways as per the Kyoto Encyclopaedia of Genes and Genomes (KEGG), and determined enrichment of pathways using Fisher's exact test (Kanehisa and Goto, 2000). In order to account for multiple testing, a Benjamini-Hochberg correction was applied (Benjamini and Hochberg, 1995). Pathways were deemed to be significant if they obtained a corrected P-value of < 0.05. Pathways specifically addressing human diseases and disorders were not included in further analysis of DAVID identified pathways as these were not relevant to this study.

2.3.8. eQTL analysis

Samples for eQTL analysis were obtained from CH and Holstein-Friesian cattle that had been genotyped as part of the current study and for which RNA-Seq data were available within our group. The RFI range of the CH and Holstein-Friesian cattle were 1.48 to -0.98 and 1.48 to -1.41 respectively. RNA-Seq raw read counts were collated from liver and muscle tissue analyses carried out by our group in published studies (Keogh et al., 2016a, Keogh et al., 2016b) and studies in preparation (Higgins et al., McKenna et al.) related to feed efficiency traits. Forty-two liver samples and 39 muscle samples were brought forward to eQTL analysis. For eQTL identification, liver and muscle samples were analysed separately.

Raw read counts were filtered and genes with more than 10 instances of zero expression were removed from analysis, resulting in 14,588 and 14,309 genes with expression in the liver and muscle, respectively, remaining for eQTL analysis. Filtered raw read counts were normalised using DESeq2's *variancestabilizingTransformation* (VST) command (Love et al., 2014). Covariates included in DESeq2 were batch, RFI status (i.e. high or low RFI) and breed. VST normalised counts were brought forward for eQTL analysis.

eQTL analysis was carried out using the R package Matrix eQTL (Shabalin, 2012). Only SNPs that reached genome-wide significance after meta-analysis (n=24) and their nearest gene were considered for eQTL analysis. If a nearest gene was greater

than 100 kilobases away from a SNP, this combination was not included in eQTL analysis. As part of eQTL analysis RFI, breed and sex were included as covariates. SNPs with a MAF of less than 0.1 or with known functions, e.g. missense and synonymous mutations, were excluded, as were genes that were not expressed in either tissue of interest, i.e. liver or muscle. This resulted in 11 SNPs in the analysis. A Bonferroni correction was applied to account for the 11 SNPs. If an eQTL reached a *P*-value of 0.0045 or less, it was considered significant after multiple test correction.

2.4. Results

2.4.1. GWAS and meta-analysis for RFI

GWAS results generated for RFI by meta-analysis are plotted in Figure 2.1. Seven SNPs achieved genome-wide significance for RFI (Table 2.1). The SNP most associated with RFI was rs43555985, located at chromosome 8 position 69,658,202, a non-coding region 53.4kb upstream from *GFRA2*. Two variants were within the start-stop coordinates of genes; intronic variant rs110418027 in *SMC1B* and 3' untranslated region (UTR) variant rs43691372 in *DIS3*. The remaining four SNPs are located in the non-coding region of the bovine genome and their distance to nearest gene is specified in Table 2.1. The per breed GWAS results for each of these RFI associated variants are illustrated in Table 2.2 and Supplementary Table 2.4. Functional annotation of genes containing or near to nominally significant SNPs for RFI using DAVID did not identify any enriched pathways that survived Benjamini-Hochberg correction (Benjamini and Hochberg, 1995).

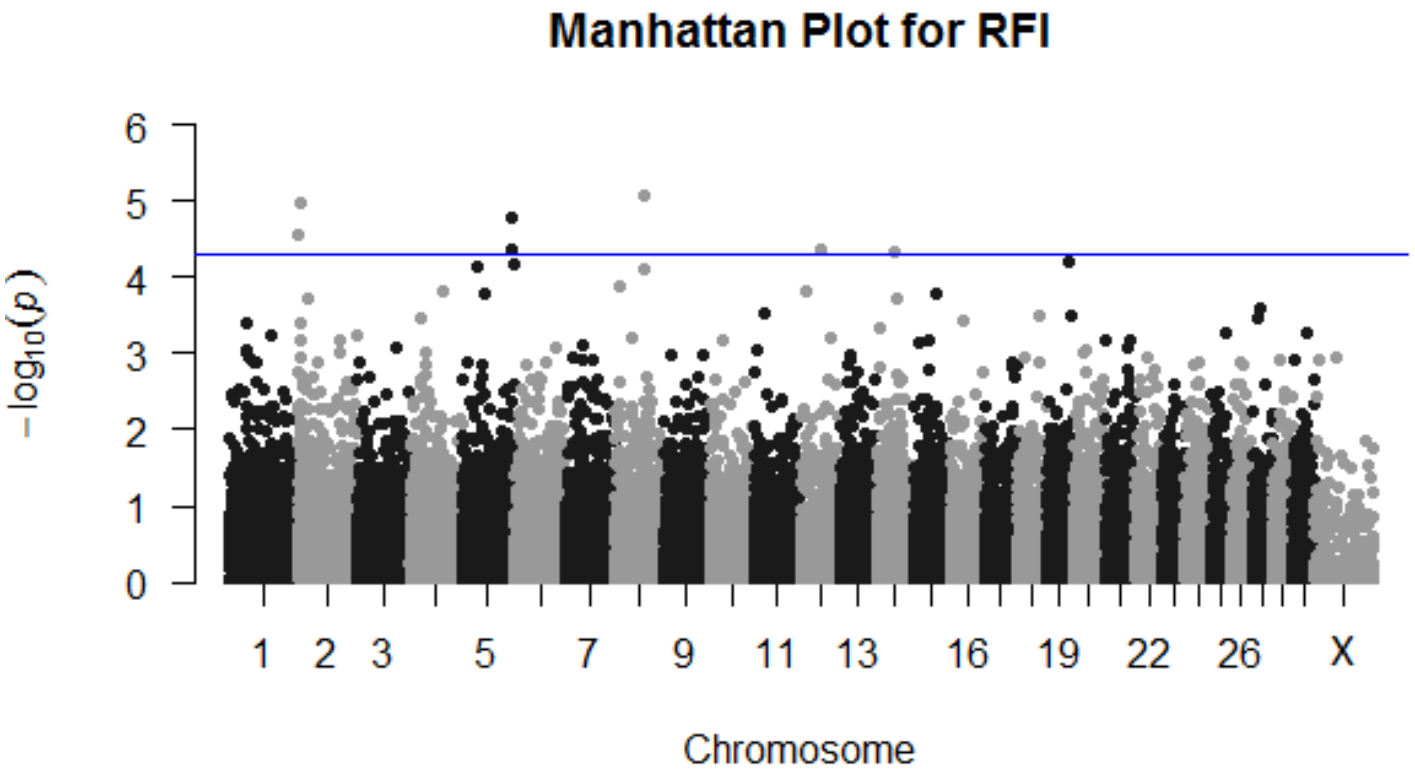


Figure 2.1. Manhattan plot represents meta-analysis results for RFI, which combined GWAS carried out for five cohorts of Irish beef cattle. The blue line indicates *P*-value<$5x10^{-5}$.

Table 2.1. SNPs which reached significance ($P<5x10^{-5}$) in a multi-breed population of beef cattle after meta-analysis of GWAS results for each respective trait.

SNP I.D.	Trait of interest	Chr_mb	Zscore	P-value	Nearby gene	SNP location relative to gene
rs43555985	RFI	8_69	-4.458	8.28E-06	GFRA2	53.4kb upstream
rs41638273	RFI	2_6	4.4	1.08E-05	SLC40A1	15.7kb upstream
rs109695205	RFI	5_113	4.313	1.61E-05	NFAM1	26.5kb upstream
rs110161277	RFI	2_2	4.192	2.76E-05	PLEKHB2	143.8kb downstream
rs110418027	RFI	5_116	-4.089	4.34E-05	SMC1B	Intron variant
rs43691372	RFI	12_47	4.082	4.47E-05	DIS3	3' UTR variant
rs42820242	RFI	14_44	4.081	4.48E-05	IL7	104.2kb downstream
rs386023985	ADG	19_48	-6.593	4.32E-11	ERN1	7.9kb upstream
rs135897656	ADG	3_119	6.195	5.83E-10	CSF2RA	Intron variant
rs136457441	ADG	19_28	5.936	2.93E-09	RPL26	Missense variant
rs110660154	ADG	1_19	5.314	1.08E-07	SPATA16	265kb downstream
rs110780286	ADG	18_15	4.492	7.06E-06	ITFG1	Intron variant
rs382426807	ADG	19_43	4.473	7.70E-06	STAT5A	Synonymous variant
rs41595251	ADG	9_91	-4.375	1.22E-05	OPRM1	269kb upstream
rs110590483	ADG	11_39	-4.243	2.21E-05	CCDC85A	509kb downstream
rs109252082	ADG	19_53	4.124	3.72E-05	TBC1D16	Intron variant
rs41592667	ADG	9_35	4.12	3.78E-05	FRK	88kb downstream
rs41630180	ADG	17_1	-4.097	4.18E-05	TLL1	Intron variant
rs41614223	ADG	9_27	-4.086	4.39E-05	NKAIN2	8.8kb downstream
rs137576435	ADG	19_12	-4.079	4.52E-05	BCAS3	Intron variant
rs136789347	ADG	23_52	-4.069	4.72E-05	OR5M10	13.8kb upstream
IDBV32000008978	FI	20_67	4.355	1.33E-05	ADAMTS16	Synonymous variant
rs55617218	FI	19_14	-4.205	2.61E-05	HNF1B	Intron variant
rs109691080	FI	1_6	4.084	4.43E-05	MAP3K7CL	58.9kb upstream

SNP: Single nucleotide polymorphism; RFI: Residual feed intake; ADG: Average daily gain; FI: Feed intake; Chr_mb: Chromosome_megabase

Table 2.2. Individual breed GWAS results for all genetic variants that reached genome-wide significance following meta-analysis.

Trait	SNP ID	Meta-analysis *P*-value	Direction of Effect	AA *P*-value	BB *P*-value	CH *P*-value	LM *P*-value	SI *P*-value
RFI	rs43555985	8.28E-06	-----	2.14E-01	1.18E-01	5.30E-01	1.24E-03	2.39E-03
	rs41638273	1.08E-05	+++++	1.65E-01	3.16E-03	5.79E-03	9.03E-02	1.74E-01
	rs109695205	1.61E-05	+++++	3.02E-02	4.12E-02	1.09E-01	4.04E-02	2.35E-02
	rs110161277	2.76E-05	+++++	1.68E-01	5.24E-03	1.41E-02	1.50E-01	8.58E-02
	rs110418027	4.34E-05	-----	7.77E-02	8.29E-02	4.74E-02	3.92E-02	7.50E-02
	rs43691372	4.47E-05	+++++	1.74E-01	5.36E-02	6.36E-01	8.67E-03	4.65E-03
	rs42820242	4.48E-05	+++++	1.49E-01	2.74E-01	7.48E-02	1.06E-02	4.41E-02
ADG	rs386023985	4.32E-11	-----	3.43E-01	3.82E-01	4.71E-04	1.16E-07	1.30E-03
	rs135897656	5.83E-10	+++++	1.54E-01	1.70E-01	3.64E-07	1.19E-06	3.01E-01
	rs136457441	2.93E-09	+-+++	4.58E-01	-9.64E-01	4.55E-06	1.88E-05	1.03E-02
	rs110660154	1.08E-07	+++++	3.69E-02	-3.56E-01	5.20E-05	9.81E-02	5.63E-03
	rs110780286	7.06E-06	+++++	2.13E-01	-9.79E-03	3.94E-01	9.86E-02	4.21E-04
	rs382426807	7.70E-06	+-+-+	5.84E-01	-7.69E-01	1.50E-07	5.65E-06	6.85E-01
	rs41595251	1.22E-05	-----	1.83E-01	-9.94E-01	1.17E-01	4.61E-05	1.70E-02
	rs110590483	2.21E-05	-----	3.60E-01	1.69E-01	5.64E-03	5.12E-01	2.27E-03
	rs109252082	3.72E-05	+++++	8.27E-02	5.49E-01	1.26E-03	5.09E-01	1.07E-02
	rs41592667	3.78E-05	+++++	4.92E-01	-6.02E-01	7.18E-03	8.14E-02	6.75E-03
	rs41630180	4.18E-05	-----	8.08E-01	-1.87E-03	1.25E-01	6.19E-04	2.61E-01
	rs41614223	4.39E-05	-----	2.94E-01	-7.84E-03	9.24E-04	6.27E-02	5.16E-01
	rs137576435	4.52E-05	-----	3.95E-01	4.11E-01	2.03E-02	7.64E-01	1.66E-04
	rs136789347	4.72E-05	-----	1.99E-01	6.66E-01	1.35E-01	1.77E-04	5.03E-02
FI	IDBV32000008978	1.33E-05	++++-	6.00E-03	1.69E-03	7.68E-03	3.35E-02	8.59E-01
	rs55617218	2.61E-05	+----	4.22E-01	1.75E-01	2.83E-01	1.92E-04	8.14E-03
	rs109691080	4.43E-05	-++++	6.89E-01	4.45E-01	1.41E-04	1.88E-01	6.53E-03

SNP: single nucleotide polymorphism; RFI: residual feed intake; ADG: average daily gain; Direction of effect: direction of effect of the Illumina A allele; FI: feed intake; AA: Aberdeen Angus; BB: Belgian Blue; CH: Charolais; LM: Limousin; SI: Simmental.

2.4.2. GWAS and meta-analysis for ADG

GWAS results for ADG are illustrated in Figure 2.2. A total of 14 SNPs reached genome-wide significance for ADG. The most significantly associated SNP was rs386023985 which is located at chromosome 19 position 48,916,589, 7.9kb upstream from the *ERN1* gene. One missense variant, rs136457441 in *RPL26*, was associated with ADG. One associated variant was synonymous, rs382426807 in *STAT5A*. Five intronic variants were associated with ADG in the genes: *CSFRA2, ITFG1, TBC1D16, TLL1* and *BCAS3*. The remaining 5 associated SNPs were located upstream or downstream of genes as indicated in Table 2.1. Individual breed GWAS results for the SNPs associated with ADG following meta-analysis are outlined in Table 2.2 and Supplementary Table 2.4.

Functional annotation of genes nearest to nominally significant SNPs for ADG identified 7 pathways that were significantly enriched following Benjamini-Hochberg correction (Benjamini and Hochberg (1995), Table 2.3). The thyroid hormone signalling pathway was the most enriched pathway (corrected P=0.01).

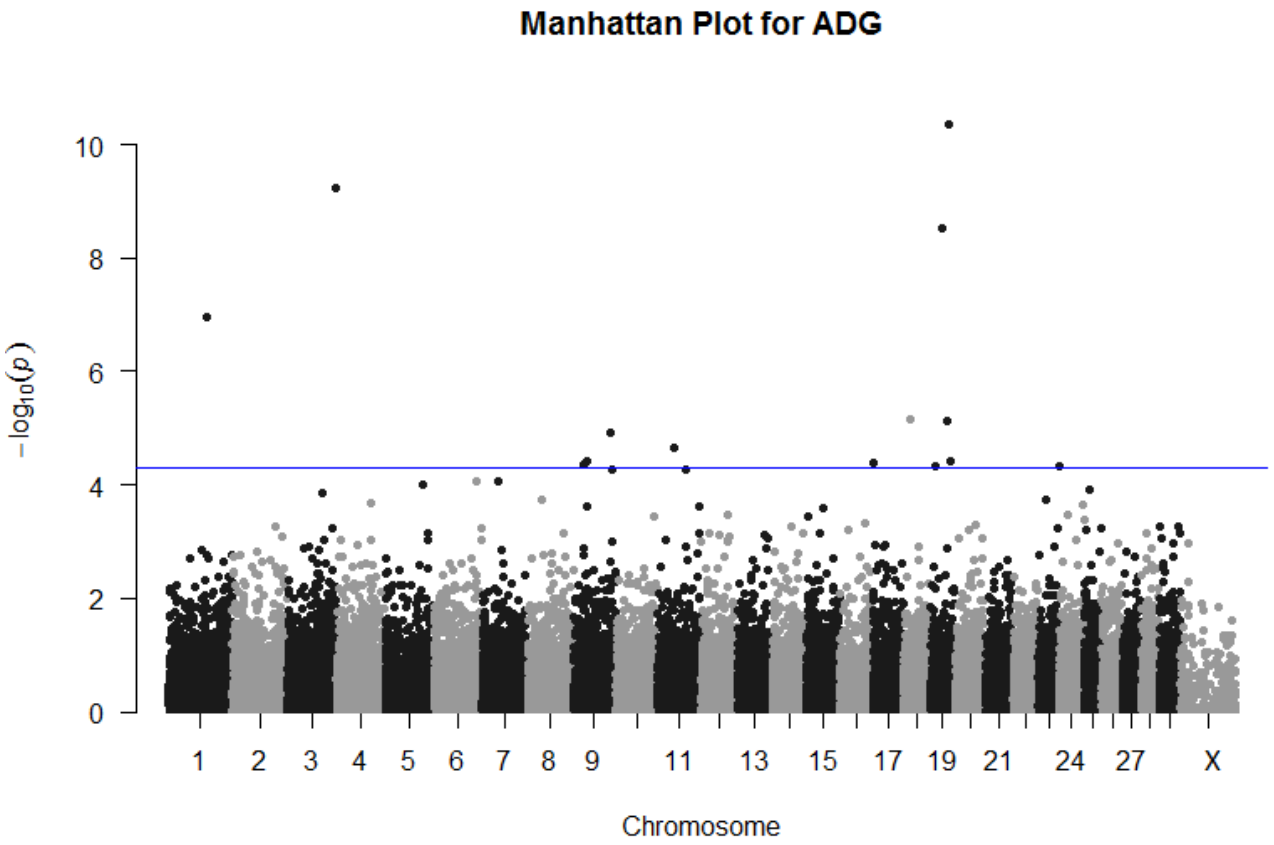


Figure 2.2. Manhattan plot of meta-analysis results for ADG. Meta-analysis was carried out on GWAS results generated for five breeds of Irish beef cattle. The blue line indicates *P*-value<5x10-5.

Table 2.3. Significant KEGG pathways identified for each trait in a multi-breed population of beef cattle following meta-analysis of GWAS results.

Trait	Biological Process	B-H *P*-value	Number of genes
ADG	Thyroid hormone signalling pathway	0.010	20
ADG	cGMP-PKG signalling pathway	0.011	25
ADG	Vascular smooth muscle contraction	0.015	18
ADG	Retrograde endocannabinoid signalling pathway	0.013	18
ADG	Focal adhesion	0.027	27
ADG	cAMP signalling pathway	0.026	26
ADG	Adherens junction	0.029	13
FI	Axon guidance	0.001	23
FI	Thyroid hormone signalling pathway	0.015	19

B-H *P*-value: Benjamini-Hochberg corrected P-value; ADG: average daily gain; FI: feed intake. Pathways were designated as significant if they reached Benjamini-Hochberg corrected P<0.05.

2.4.3. GWAS and meta-analysis for FI

GWAS results for FI are plotted in Figure 2.3. Three SNPs reached genome-wide significance for FI (Table 2.1). Individual breed GWAS results for these variants are presented in Table 2.2 and Supplementary Table 2.4. The SNP most associated with FI was IDBV32000008978 located at chromosome 20 position 67,944,737. This is a synonymous variant in *ADAMTS16*. The other two were an intronic variant in *HNF1B* and a variant located 58.9kb upstream of MAP3K7CL. Functional annotation of the FI SNP results identified two pathways that were significantly enriched after correction; axon guidance and the thyroid hormone signalling pathway (Table 2.3).

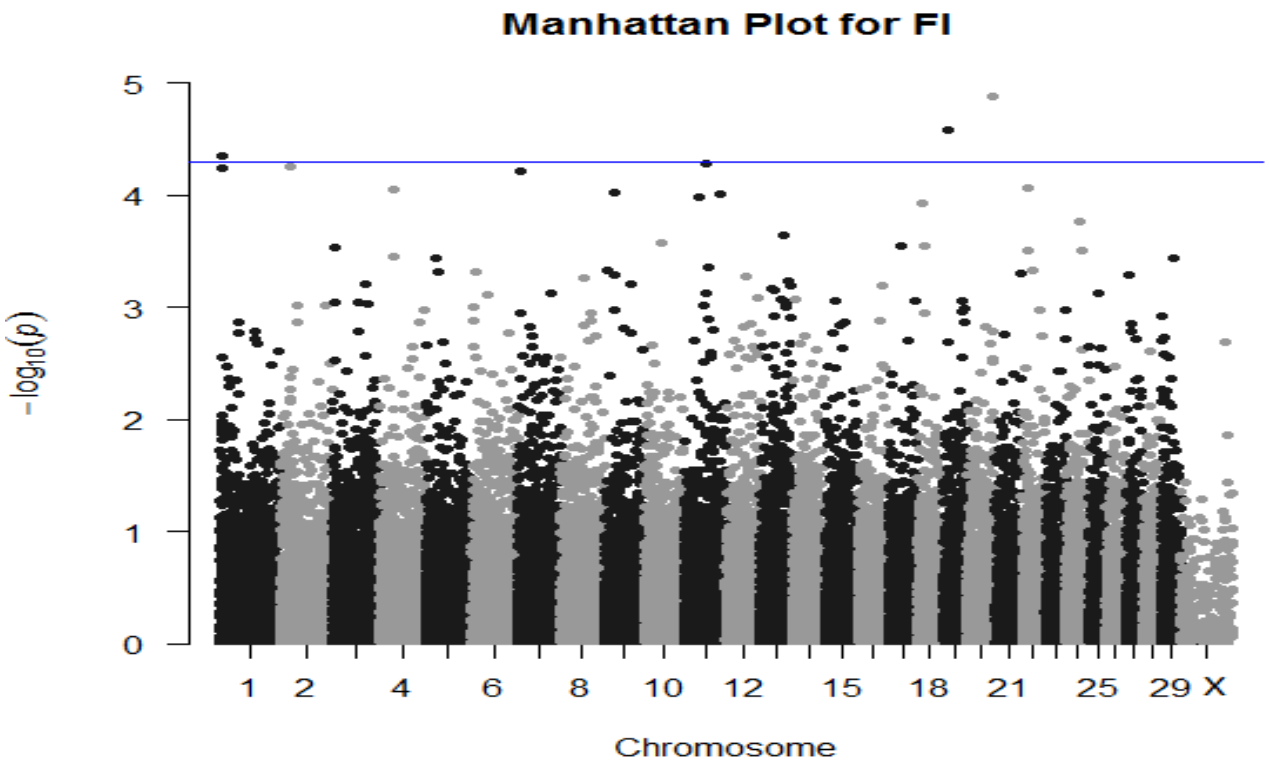


Figure 2.3. Manhattan plot of FI meta-analysis of GWAS results for Irish beef cattle. The blue line indicates *P*-value<5x10-5.

2.4.4. Validation of internationally identified SNPs in Irish cattle

Of the 102 internationally identified RFI SNPs included on the custom IDBv3 genotyping chip, 71 passed all QC measures and were included in the GWAS for RFI in the current study. Two of these SNPs, rs29014641 and rs109500421, were nominally significant in our study but did not survive multiple test correction for this subset of SNPs. This subset of SNPs was not exhaustive for RFI and did not include all variants within quantitative trait loci (QTLs) as identified by Nkrumah et al. (2007), for example. However, a *post-hoc* search for genotyped SNPs within those regions found no genetic variant reached genome-wide significance following meta-analysis within these QTLs.

2.4.5. eQTL analysis of SNPs identified as significant from meta-analysis

Table 2.4 contains results of eQTL analysis. One cis-eQTL was detected in liver, between rs43555985, the top associated SNP from the RFI GWAS, and *GFRA2* (P=0.0038; survives multiple test correction). eQTL analysis indicated that the minor allele of rs4355985 is associated with increased expression of *GFRA2* (Figure 2.4). The same minor allele is associated with lower RFI in the GWAS. The effect of *GFRA2* expression on RFI is presented in Supplementary Figure 2.1 on a per genotype basis.

Table 2.4. Results from eQTL analysis of genome-wide significant SNPs in liver and muscle.

SNP	Nearest Gene	Trait	Liver *P*-value	Muscle *P*-value
rs43555985	*GFRA2*	RFI	**0.0038***	0.25
rs109695205	*NFAM1*	RFI	0.95	0.55
rs110418027	*SMC1B*	RFI	0.15	Not expressed
rs43691372	*DIS3*	RFI	0.96	0.16
rs386023985	*ERN1*	ADG	0.99	0.23
rs110780286	*ITFG1*	ADG	0.83	0.23
rs382426807	*STAT5A*	ADG	0.94	0.72
rs41592667	*FRK*	ADG	0.22	0.19
rs41630180	*TLL1*	ADG	Not expressed	0.33
rs137576435	*BCAS3*	ADG	0.25	0.93
rs109691080	*MAP3K7CL*	FI	Not expressed	0.80

SNP: Single nucleotide polymorphism; ADG: average daily gain; FI: feed intake; RFI: residual feed intake, *: eQTLs were designated as significant if they reached $P<0.0045$

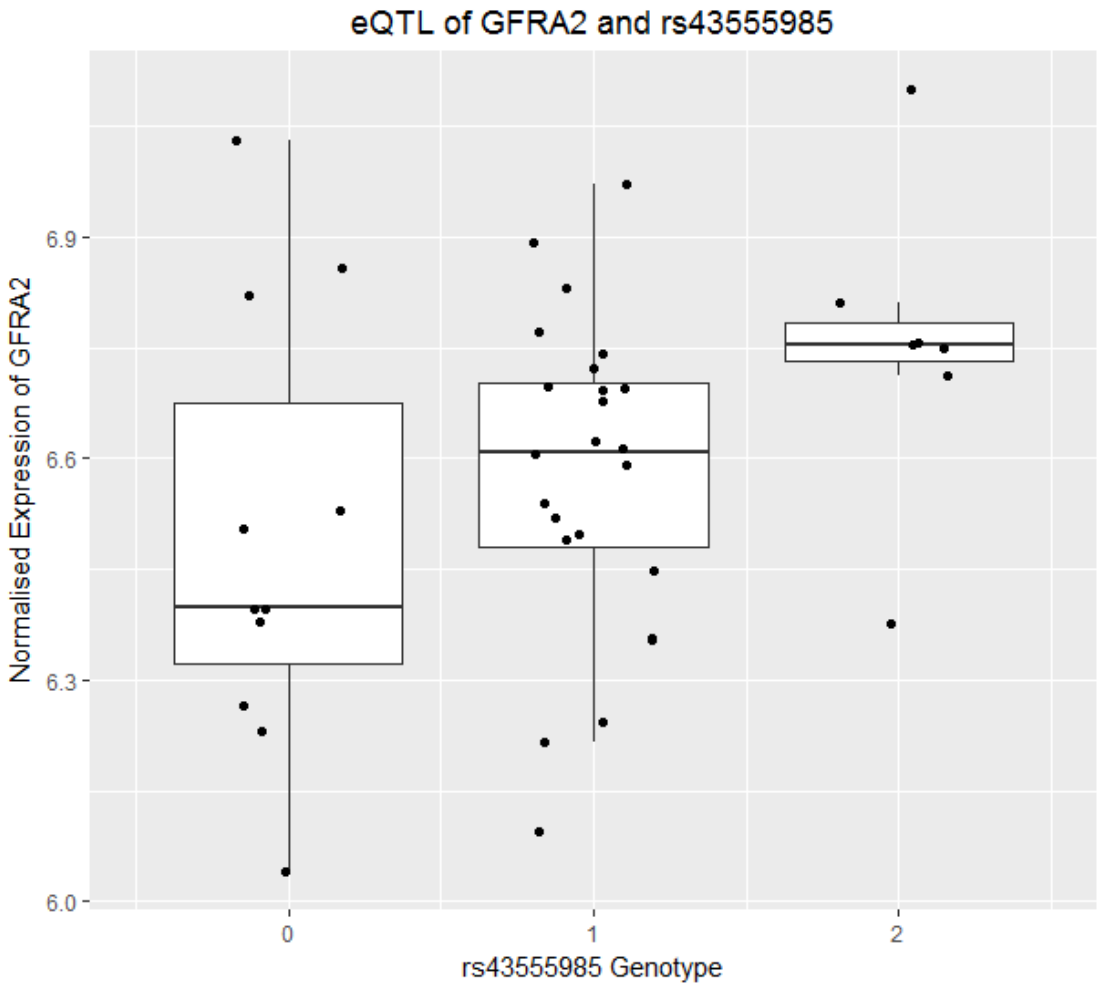


Figure 2.4. Boxplot representing the relationship between rs43555985 genotypes and normalised liver gene-expression of *GFRA2*. Presence of the minor allele of rs43555985 is correlated with increased expression of *GFRA2*. 0: GG; 1: GA; 2: AA.

2.5. Discussion

Despite the economic and environmental benefits of RFI, the trait or indeed any measure of feed efficiency, is not widely adopted within breeding programmes for beef cattle due to the difficulty and expense associated with measuring feed intake (Hayes et al., 2013). The identification of robust genetic markers of RFI applicable to several breeds, as well as crossbred cattle, would enable the traits inclusion in genomic breeding programmes. This study sought to identify SNPs associated with RFI that could be applicable to Irish beef production enterprises as well as

uncovering novel markers of potential use to international beef producers. To unravel the underlying biology causing phenotypic variation in feed efficiency related traits, we carried out eQTL analysis of GWAS-identified variants to study their effect on local gene expression.

rs43555985 was associated with RFI and is an eQTL of the *GFRA2* gene in liver tissue. The minor allele of this SNP was associated lower RFI within each of the individual breed GWAS and following meta-analysis. The minor allele of rs43555985 was also associated with increased expression of *GFRA2* following eQTL analysis. *GFRA2* is a cell-surface receptor that facilitates binding of a member of the glial cell-derived neurotrophic factor family. *GFRA2* knock-out mice are unable to digest food correctly, have impaired salivary secretion and gut motility and exhibit a slower growth rate than wild-type mice while having an increased basal metabolic rate (Rossi et al., 2003). If increased *GFRA2* expression is associated with improved feed efficiency, the mechanism may involve lowering metabolic rates. Increased metabolic rate leads to increased energy requirements to carry out biological processes and to maintain physiological homeostasis, resulting in less consumed energy being used for growth (Herd and Arthur, 2009). It has been illustrated previously that high-RFI lambs have a higher basal metabolic rate than their low-RFI (more desirable) counterparts and low-RFI heifers exhibited lower metabolic rates than their high-RFI (inefficient) counterparts (Zhang et al., 2017, Gonano et al., 2014). Further investigation and validation of rs43555985 prior to inclusion in genomic breeding programmes is required. Furthermore, rs43555985 is also located 79.9kb upstream from *XPO7*. *Post-hoc* eQTL analysis for this gene illustrated that there is no statistically significant relationship between rs43555985 genotype and *XPO7* expression.

The second most statistically significant SNP for RFI, rs41638273, maps to a region of chromosome 2 that contains the *SLC40A1* gene. This region is also the site of a QTL for RFI which contains the myostatin gene (Saatchi et al., 2014b). Specific mutations in the myostatin gene have been linked with increased muscle growth traits (Grobet et al., 1997). Improved feed efficiency was associated with double

muscled Angus steers by Cafe et al. (2014) when compared to lesser muscled counterparts. *DIS3*, a gene nearby to a variant associated with RFI in the current study, encodes a protein involved in RNA metabolism (Dziembowski et al., 2007) and has been linked with feed conversion efficiency in pigs (Horodyska et al., 2017).

The minor allele of rs386023985 was negatively associated with ADG following GWAS meta-analysis in the current study. Similarly, this variant was negatively associated with ADG within each individual breed GWAS conducted. This SNP also reached genome-wide significance within the LM individual breed GWAS. rs386023985 has not previously been associated with ADG or other growth traits in cattle. The gene nearest to rs386023985 is *ERN1 (IRE1)*, a sensor of metabolic stress, is involved in the unfolded protein response (Shan et al., 2017).

Copy number variation in *RPL26*, a ribosomal protein gene, has been linked to RFI divergence in Holstein cows (Hou et al., 2012). A variant identified in this study, rs136457441, is a missense variant in *RPL26* causing an isoleucine to threonine change at amino acid position 67 in the RPL26 protein. This variant, associated with ADG following meta-analysis in the current study and reached genome-wide significance for ADG within the CH individual breed GWAS, is located in exon 3 of *RPL26*. rs136457441 has been designated as tolerated by the Sorting Tolerant from Intolerant (SIFT) algorithm, which predicts whether amino acid substitutions effect protein function (Kumar et al., 2009). Further investigation into the functional effect of this mutation is required to elucidate its biological role in ADG.

An exonic variant associated with ADG is rs382426807, a synonymous variant in *STAT5A*. This gene encodes a transcription factor that can be activated as part of the somatotropic axis, which is the pathway involved in the secretion of growth hormone and skeletal muscle growth (Renaville et al., 2002). *STAT5A* has been associated with increased live weight gain in Polish Black-and-White bulls (Oprządek and Flisikowski, 2003) and increased expression of the growth hormone receptor, an

upstream activator of STAT5A, has been previously demonstrated in efficient beef heifers by Kelly et al. (2013).

Five variants associated with ADG were located in introns of the following genes: *TLL1, CSF2RA, ITFG1, TBC1D16* and *BCAS3*. *TLL1* encodes a member of the tolloid family metalloproteases that have been previously implicated in the cleavage and development of myostatin in humans (Lee, 2008). Myostatin in its normal state negatively regulates muscle growth. The production of aberrant myostatin protein isoforms results in the development of the double muscle phenotype (Grobet et al., 1997).

CSF2RA encodes for a granulocyte/macrophage colony stimulating factor (Suzuki et al., 2008). *ITFG1,* the gene within which rs110780286 is located, is involved in T-cell differentiation and may induce the production of anti-inflammatory cytokines (Fiscella et al., 2003). It has been previously illustrated that immune genes and immune pathways are associated with variation in feed efficiency and ADG in cattle (Reynolds et al., 2017, Alexandre et al., 2015). Several groups have suggested that the immune system plays a key role in weight gain and feed efficiency in cattle. For example, Reynolds et al. (2017) found that steers with higher ADG have lower immunity related gene expression and it has been demonstrated that cattle with poor feed efficiency had increased activation of their immune system (Alexandre et al., 2015). It is possible that cattle with poor feed efficiency and low ADG are experiencing chronic inflammation which results in poor feed efficiency which has been suggested previously by Alexandre et al. (2015) following analysis of beef cattle divergent in RFI and by Mani et al. (2013) upon investigation of inflammation in RFI divergent pigs.

rs41595251 is associated with ADG and is a variant located upstream from *OPRM1,* the μ-opioid receptor gene, on chromosome 9. *OPRM1* has been associated with increased food intake in humans (Davis et al., 2009). ADG-associated SNP rs136789347 is nearby to *OR5M10* which encodes for an olfactory receptor in

humans (Tobar et al., 2009). Olfactory receptors have been suggested as one method by which the endocannabinoid system stimulates the feeding drive in mice (Soria-Gómez et al., 2014). rs41614223 is located downstream from the transcriptional start site of *NKAIN2*, which produces a Sodium-Potassium ATPase involved in action potential generation in neurons (Gorokhova et al., 2007). Each of these genes, *OPRM1, OR5M10* and *NKAIN2* have a neurological function. There is evidence from bovine studies (Perkins et al., 2014, Alam et al., 2012) that there is significant neurological control of food consumption. The association of these neurological genes with ADG in this cohort of beef cattle may further indicate that feeding behaviour in cattle may also be subject to some degree of neurological control (Fitzsimons et al., 2017). Further investigation is required to investigate the role neurological systems play in modulating the development of divergent RFI and related traits in cattle.

rs41592667 is upstream of *FRK* which encodes for tyrosine-protein kinase FRK. Gene sets enriched for cell cycle-related genes, similar to *FRK*, have previously been shown to be associated with feed intake and feed efficiency in beef cattle (Sherman et al., 2010). *TBC1D16*, a gene which encodes for a GTPase and contains the intronic variant rs109252082, has been associated with growth rate in pigs (Puig-Oliveras et al., 2014). Despite these SNPs not being associated with feed efficiency or component traits prior to the current study, they are near to, or within, genes that have been associated with feed efficiency related traits previously.

HNF1B, nearby to a variant associated with FI, has previously been identified as differentially expressed in Holstein cattle divergent for RFI (Xi et al., 2015), and this gene is a target of miR-802, which has been identified as upregulated in high RFI cattle (Al-Husseini et al., 2016). The silencing of *HNF1B* in mice leads to impaired insulin sensitivity (Kornfeld et al., 2013). However, previous work by our group has shown that RFI divergent beef cattle have similar levels of insulin sensitivity and it is unlikely that insulin sensitivity plays a role in RFI divergence (Fitzsimons et al., 2014c). Further work is required to understand the contribution of *HNF1B* to the development of divergence in FI. In this study the variant IDBV332000008978 was

associated with FI. This variant is a synonymous variant within the *ADAMTS16* gene which is a member of ADAMTS protease family and has previously been identified as associated with FCR in pigs (Sahana et al., 2013).

Following functional gene set enrichment analysis using DAVID, the thyroid hormone signalling pathway was found to be most enriched for ADG. Thyroid hormones play a key role in the regulation of basal metabolism in mammals (Mullur et al., 2014), although it has been demonstrated previously that the levels of thyroid hormones are not related to RFI status in heifers (Kelly et al., 2010b). However, in a study of dairy cattle is was reported that low levels of thyroid hormones are associated with lower RFI (Dechow et al., 2017). Further investigation into the role of the thyroid hormone signalling pathway is warranted to further elucidate the role this biological mechanism plays in the divergence of RFI in cattle. The retrograde endocannabinoid signalling pathway was also found to reach significance level in the list of nominal significant genes for ADG. It has been demonstrated that the endocannabinoid system plays a role in inducing food intake and modulating energy expenditure and feed intake in mice (Jo et al., 2005, Matias and Di Marzo, 2007). It is possible that alterations in genes in the retrograde endocannabinoid pathway may also stimulate or inhibit feeding behaviours in cattle which may impact on feed efficiency. It has been observed previously that low RFI cattle have fewer daily feeding events and have a lower eating rate than high RFI cattle (Kelly et al., 2010a). Focal adhesion was another pathway found to be enriched for nominally significant ADG associated SNPs. Focal adhesion is a pathway involved in cell motility, proliferation and survival. This pathway is dependent upon focal adhesion kinase (Zachary, 1997). *PTK2*, the gene encoding for focal adhesion kinase has been previously noted as downregulated in high-RFI animals from a population of dairy cattle (Salleh et al., 2017).

2.6. Conclusion

In this study we illustrate genome-wide associations between SNPs and RFI and its component traits in beef cattle. In total, we identified 24 SNPs as reaching statistical significance for RFI, ADG and FI in a multi-breed cohort of beef cattle. Several of

the SNPs identified in this study are located nearby or within genes related to immune function, muscle growth and development, and neurological pathways. The identification of a novel eQTL for RFI at *GFRA2* also represents an insight into the biology of feed efficiency.

Due to the small sample size of our individual breed GWAS, which we used meta-analysis to overcome, all identified SNPs and the eQTL must be validated, both in larger Irish and international populations before incorporation into genomic assisted beef cattle breeding programmes. Furthermore, validation is required in larger reference populations to account for the LD and genetic heterogeneity which exists between breeds of cattle.

An additional method which may have been employed to increase sample size could have been single-step GWAS (ssGWAS) (Wang et al., 2014, Wang et al., 2012, Lu et al., 2018). ssGWAS incorporates genotypes, phenotypes and pedigree information to calculate genomic estimated breeding values for animals with or without genotypes (Wang et al., 2014).

It is important to ensure that the SNPs influence these traits and have no negative impact on other economically important production traits. SNPs with a validated desirable effect can be included in Irish and international genomic assisted breeding programmes to facilitate the rapid and cost effective selection of more feed efficient beef cattle.

2.7. Supplementary information

Supplementary Table 2.1. The names and location of RFI related SNPs which are included in the IDBv3 genotyping chip.

Chromosome	SNP name (rs)	Citation
1	rs42548511	Rolf et al., 2012
1	rs43266121	Rolf et al., 2012
1	rs109384003	Rolf et al., 2012
2	rs110073925	Rolf et al., 2012
2	rs109949037	Rolf et al., 2012
2	rs110211659	Rolf et al., 2012
2	rs109095895	Rolf et al., 2012
2	rs42320097	Serao et al., 2013
2	rs29019569	Sherman et al., 2008
3	rs207913429	Alexandre et al., 2014
3	rs41585097	Nkrumah et al., 2007; Rolf et al., 2012
3	rs42936243	Rolf et al., 2012
4	rs109406059	Rolf et al., 2012
4	rs42824767	Rolf et al., 2012
4	rs41654149	Serao et al., 2013
5	rs110188299	Nkrumah et al., 2007; Rolf et al., 2012
5	rs109158476	Serao et al., 2013
5	rs43706834	Sherman et al., 2008
6	rs109373082	Barendse et al., 2007; Rolf et al., 2012
6	rs43455987	Rolf et al., 2012
6	rs109201532	Rolf et al., 2012
6	rs109452133	Serao et al., 2013
6	rs41663978	Serao et al., 2013
6	rs43453950	Serao et al., 2013
7	rs110202648	Rolf et al., 2012
8	rs136892391	Abo-Ismail et al., 2014
8	rs110937563	Rolf et al., 2012
8	rs109380245	Rolf et al., 2012
8	rs29022959	Rolf et al., 2012
8	rs41661176	Rolf et al., 2012
8	rs109500421	Serao et al., 2013
8	rs110922588	Serao et al., 2013
9	rs42797639	Rolf et al., 2012
9	rs110608668	Rolf et al., 2012
9	rs41610951	Rolf et al., 2012
9	rs42378531	Serao et al., 2013
10	rs109635380	Rolf et al., 2012
10	rs41597140	Rolf et al., 2012
10	rs29021916	Sherman et al., 2008
11	rs41624451	Rolf et al., 2012
11	rs109589152	Rolf et al., 2012

Chromosome	SNP name (rs)	Citation
11	rs109280551	Rolf et al., 2012
11	rs41256074	Serao et al., 2013
12	rs110643661	Nkrumah et al., 2007; Rolf et al., 2012
12	rs41614805	Rolf et al., 2012
12	rs41626249	Rolf et al., 2012
12	rs42456314	Serao et al., 2013
14	rs109272278	Rolf et al., 2012
14	rs110442376	Rolf et al., 2012
14	rs110371924	Rolf et al., 2012
15	rs41755948	Abo-Ismail et al., 2014
15	rs208805443	Abo-Ismail et al., 2014
15	rs109918426	Barendse et al., 2007; Rolf et al., 2012
15	rs41759150	Rolf et al., 2012
15	rs41660789	Serao et al., 2013
16	rs41820824	Abo-Ismail et al., 2014
16	rs41821600	Abo-Ismail et al., 2014
16	rs41662390	Rolf et al., 2012
16	rs41634631	Serao et al., 2013
17	rs41636597	Nkrumah et al., 2007; Rolf et al., 2012
17	rs109762011	Rolf et al., 2012
17	rs109762073	Rolf et al., 2012
17	rs110479395	Serao et al., 2013
17	rs111010038	Serao et al., 2013
17	rs29024448	Serao et al., 2013
17	rs41854727	Serao et al., 2013
18	rs134420976	Abo-Ismail et al., 2014
18	rs41639029	Rolf et al., 2012
18	rs41856111	Serao et al., 2013
19	rs41914675	Abo-Ismail et al., 2014
19	rs108970074	Rolf et al., 2012
19	rs41907619	Rolf et al., 2012
19	rs109672208	Rolf et al., 2012
20	rs43461171	Rolf et al., 2012
20	rs110275805	Rolf et al., 2012
20	rs41591215	Rolf et al., 2012
20	rs41613438	Rolf et al., 2012
20	rs43238631	Serao et al., 2013
20	rs29021101	Sherman et al., 2008
20	rs29014641	Sherman et al., 2008
21	rs43020736	Abo-Ismail et al., 2014
21	rs43020769	Abo-Ismail et al., 2014
21	rs41973640	Nkrumah et al., 2007; Rolf et al., 2012
21	rs41980271	Rolf et al., 2012
22	rs41620834	Rolf et al., 2012
22	rs110978254	Rolf et al., 2012

Chromosome	SNP name (rs)	Citation
22	rs29013532	Rolf et al., 2012
22	rs108942504	Serao et al., 2013
23	rs109404118	Rolf et al., 2012
23	rs41617133	Rolf et al., 2012
24	rs109863480	Serao et al., 2013
25	rs109899690	Rolf et al., 2012
25	rs41587267	Rolf et al., 2012
27	rs110763390	Rolf et al., 2012
28	rs109561809	Abo-Ismail et al., 2014
28	rs209765899	Abo-Ismail et al., 2014
28	rs110842770	Rolf et al., 2012
28	rs109417884	Rolf et al., 2012
29	rs110710999	Rolf et al., 2012
29	rs110252499	Rolf et al., 2012
29	rs29027034	Rolf et al., 2012
29	rs17872022	Sherman et al., 2008

Supplementary Table 2.2. Average age and standard deviation of cattle included in the phenotypic data file.

Group of animals	Age in days	Standard deviation, unless otherwise noted
Group 1	426	43.1
Group 2	479	15*
Group 3	299	48.3
Group 4	595	29
Group 5	450	Unavailable
Group 6	307	7.7
Group 7	283	18.3
Group 8	375	85

*: Represents Standard Error

Supplementary Table 2.3. Markers on IDBv3 that pass Quality Control.

This table is too large to reproduce in this thesis. However, it is available from this link: https://www.nature.com/articles/s41598-018-32374-6#Sec19. Filename: Supplementary Table S3.

Supplementary Table 2.4. Results of individual breed GWAS for each SNP identified as significantly associated with RFI and related traits following meta-analysis.

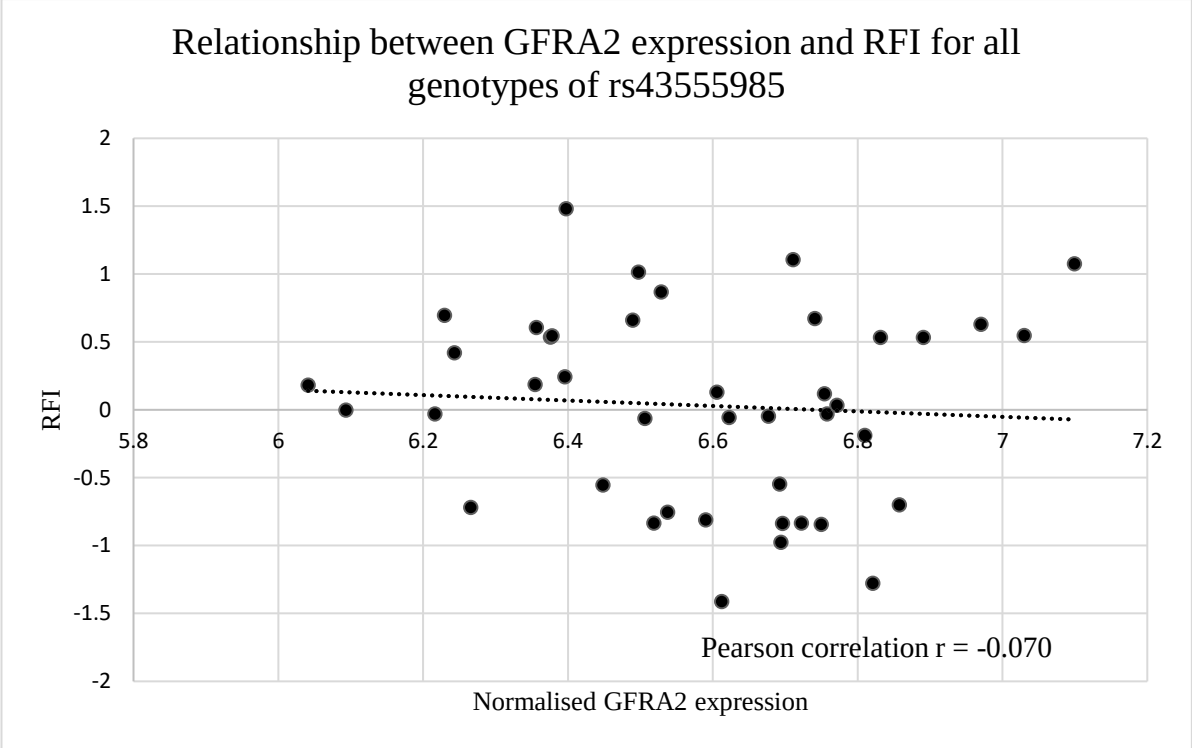
						AA				BB				CH				LM				SI			
Trait	**SNP ID**	**Nearby gene**	**Meta *P***	**Z**	**Direction**	**GWAS *P***	**Beta**	**SE**	**MAF**	**GWAS *P***	**Beta**	**SE**	**MAF**	**GWAS *P***	**Beta**	**SE**	**MAF**	**GWAS *P***	**Beta**	**SE**	**MAF**	**GWAS *P***	**Beta**	**SE**	**MAF**
RFI	rs43555985	*GFRA2*	8.28E-06	-4.5	-----	0.21	-0.19	0.16	0.25	0.12	-0.13	0.08	0.40	0.53	-0.04	0.06	0.38	0.001	-0.17	0.05	0.42	0.002	-0.22	0.07	0.30
	rs41638273	*SLC40A1*	1.08E-05	4.4	+++++	0.17	0.29	0.21	0.16	0.003	0.35	0.12	0.14	0.001	0.19	0.07	0.19	0.09	0.11	0.06	0.40	0.17	0.12	0.09	0.16
	rs109695205	*ENSBTAG00000046721*	1.61E-05	4.3	+++++	0.03	0.30	0.14	0.33	0.04	0.21	0.10	0.16	0.11	0.10	0.06	0.31	0.04	-0.13	0.07	0.23	0.02	0.20	0.09	0.20
	rs110161277	*PLEKHB2*	2.76E-05	4.2	+++++	0.17	0.24	0.17	0.15	0.005	0.39	0.14	0.08	0.01	0.25	0.10	0.08	0.15	0.13	0.09	0.08	0.09	0.15	0.09	0.16
	rs110418027	*SMC1B*	4.34E-05	-4.1	-----	0.07	-0.27	0.15	0.28	0.08	-0.15	0.09	0.25	0.05	-0.14	0.07	0.21	0.04	0.14	0.07	0.19	0.08	-0.17	0.10	0.12
	rs43691372	*DIS3*	4.47E-05	4.1	+++++	0.17	0.32	0.24	0.08	0.05	0.30	0.15	0.07	0.64	0.05	0.10	0.08	0.01	0.20	0.08	0.12	0.005	0.31	0.11	0.10
	rs42820242	*IL7*	4.48E-05	4.1	+++++	0.15	0.21	0.14	0.46	0.27	0.1	0.09	0.27	0.07	0.11	0.06	0.30	0.01	0.16	0.06	0.20	0.04	0.14	0.07	0.36
ADG	rs386023985	*ERN1*	4.32E-11	-6.6	-----	0.34	-0.14	0.15	0.17	0.38	-0.08	0.09	0.13	4.71E-04	-0.08	0.09	0.13	1.16E-07	-0.19	0.06	0.16	1.30E-03	-0.56	0.10	0.23
	rs135897656	*CSF2RA*	5.83E-10	6.2	+++++	0.15	0.17	0.12	0.23	0.17	0.09	0.06	0.25	3.64E-07	0.09	0.06	0.25	1.19E-06	0.04	0.04	0.24	0.30	0.43	0.09	0.18
	rs136457441	*RPL26*	2.93E-09	5.9	+-+++	0.46	0.15	0.20	0.07	-0.96	0.01	0.11	0.07	4.55E-06	-0.01	0.11	0.07	1.88E-05	0.19	0.07	0.09	0.01	0.51	0.12	0.15
	rs110660154	*SPATA16*	1.01E-07	5.3	+++++	0.04	0.19	0.09	0.48	-0.36	-0.05	0.05	0.45	5.20E-05	0.05	0.05	0.45	0.10	0.10	0.04	0.43	5.63E-03	0.12	0.07	0.38
	rs110780286	*ITFG1*	7.06E-06	4.5	+++++	0.21	0.14	0.11	0.43	-0.01	-0.17	0.07	0.38	0.39	0.17	0.07	0.38	0.10	0.15	0.04	0.42	4.21E-04	0.13	0.08	0.42
	rs382426807	*STAT5A*	7.70E-06	4.5	+-+-+	0.58	0.16	0.29	0.03	-0.77	0.03	0.12	0.08	1.50E-07	-0.03	0.12	0.08	5.65E-06	-0.03	0.06	0.12	0.69	0.47	0.10	0.13
	rs41595251	*OPRM1*	1.22E-05	-4.4	-----	0.18	-0.16	0.12	0.29	-0.99	4.92E-04	0.06	0.39	0.12	-4.92E-04	0.06	0.39	4.61E-05	-0.10	0.04	0.49	0.02	-0.30	0.07	0.48
	rs110590483	*CCDC85A*	2.21E-05	-4.2	-----	0.36	-0.10	0.11	0.45	0.17	-0.08	0.06	0.47	5.64E-03	-0.08	0.06	0.47	0.51	-0.13	0.04	0.47	2.27E-03	-0.05	0.08	0.34
	rs109252082	*TBC1D16*	3.72E-05	4.1	+++++	0.08	0.30	0.17	0.12	0.55	0.05	0.09	0.15	1.26E-03	0.05	0.09	0.15	0.51	0.13	0.05	0.28	0.01	0.05	0.08	0.25
	rs41592667	*FRK*	3.78E-05	4.1	+++++	0.49	0.08	0.12	0.29	-0.60	-0.03	0.06	0.39	7.18E-03	0.03	0.06	0.39	0.08	0.12	0.04	0.32	6.75E-03	0.14	0.08	0.36
	rs41630180	*TLL1*	4.18E-05	-4.1	-----	0.81	-0.03	0.12	0.26	-1.87E-03	0.19	0.06	0.47	0.13	-0.19	0.06	0.47	6.19E-04	-0.05	0.04	0.48	0.26	-0.26	0.07	0.49
	rs41614223	*ENSBTAG00000033195*	4.39E-05	-4.1	-----	0.29	-0.17	0.16	0.17	-0.01	0.46	0.17	0.03	9.24E-04	-0.46	0.17	0.03	0.06	-0.06	0.09	0.05	0.52	-0.46	0.25	0.02

	rs137576435	*BCAS3*	4.52E-05	-4.1	-----	0.39	-0.10	0.11	0.42	0.41	-0.05	0.06	0.45	0.02	-0.05	0.06	0.45	0.76	-0.15	0.04	0.44	1.66E-04	-0.02	0.08	0.43
	rs136789347	*ENSBTAT00000064488*	4.72E-05	-4.1	-----	0.20	-0.25	0.19	0.08	0.67	-0.04	0.08	0.23	0.14	-0.04	0.08	0.23	1.77E-04	-0.13	0.07	0.09	0.05	-0.35	0.09	0.19
FI	IDBV32000008978	*ADAMTS16*	1.33E-05	4.4	++++-	6.00E-03	2.28	0.81	0.27	1.69E-03	1.43	0.45	0.33	7.68E-03	0.99	0.37	0.24	0.03	0.65	0.31	0.21	0.86	-0.07	0.39	0.25
	rs55617218	*HNF1B*	2.61E-05	-4.2	+----	0.42	0.73	0.90	0.32	0.18	-1.56	1.14	0.03	0.28	0.77	-0.72	0.06	1.92E-04	-2.68	0.71	0.03	8.14E-03	-3.08	1.16	0.02
	rs109691080	*MAP3K7CL*	4.43E-05	4.1	-++++	0.69	-0.32	0.79	0.38	0.44	0.39	0.51	0.44	1.41E-04	1.35	0.35	0.27	0.19	0.40	0.30	0.22	6.53E-03	1.00	0.37	0.35

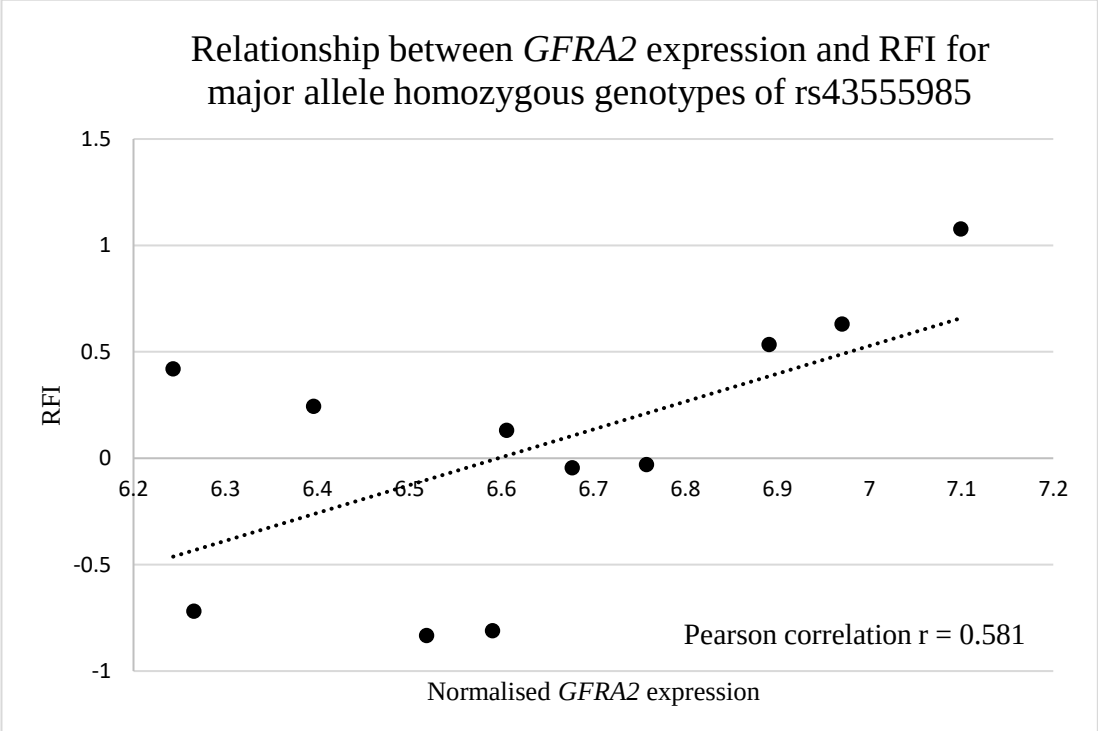
SNP: Single nucleotide polymorphism; Meta *P*: Meta-analysis *P*-value; Z: Z-score; Direction: Direction of effect of the Illumina A allele; GWAS *P*: Individual breed genome-wide association study *P*-value; Beta: Regression beta; SE: Standard error; MAF: Minor allele frequency; AA: Aberdeen Angus; BB: Belgian Blue; CH: Charolais; LM: Limousin; SI: Simmental.

Supplementary Figure 2.1. The relationship between *GFRA2* expression and RFI for genotypes of rs43555985

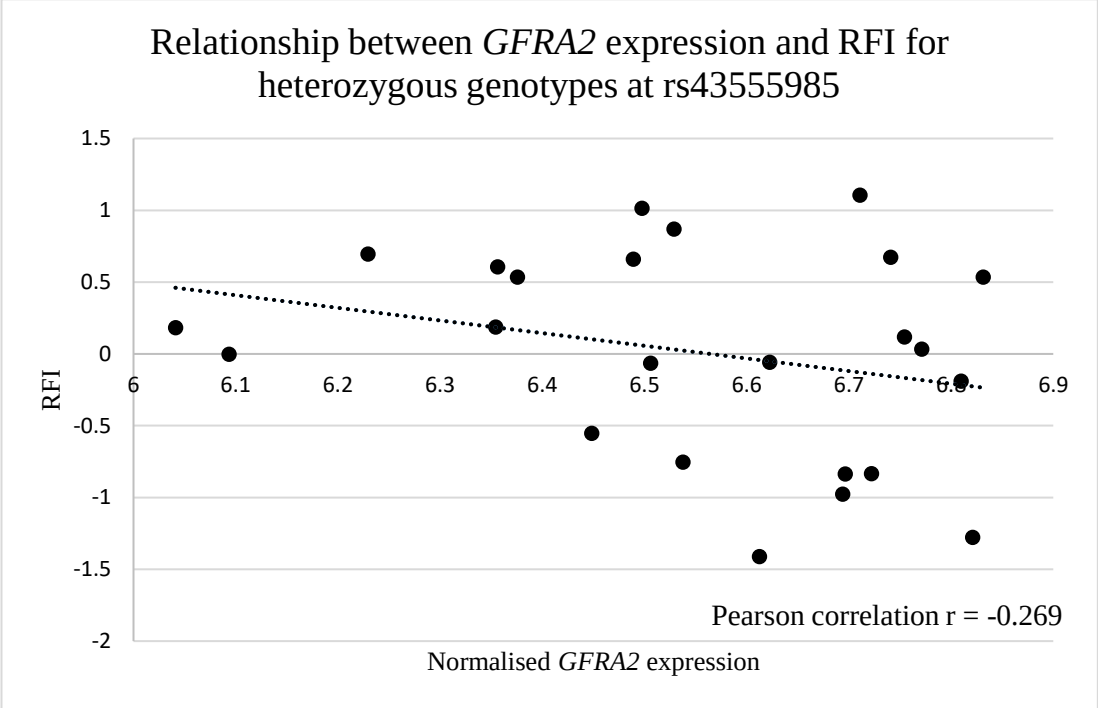
Supplementary Figure 2.1a.



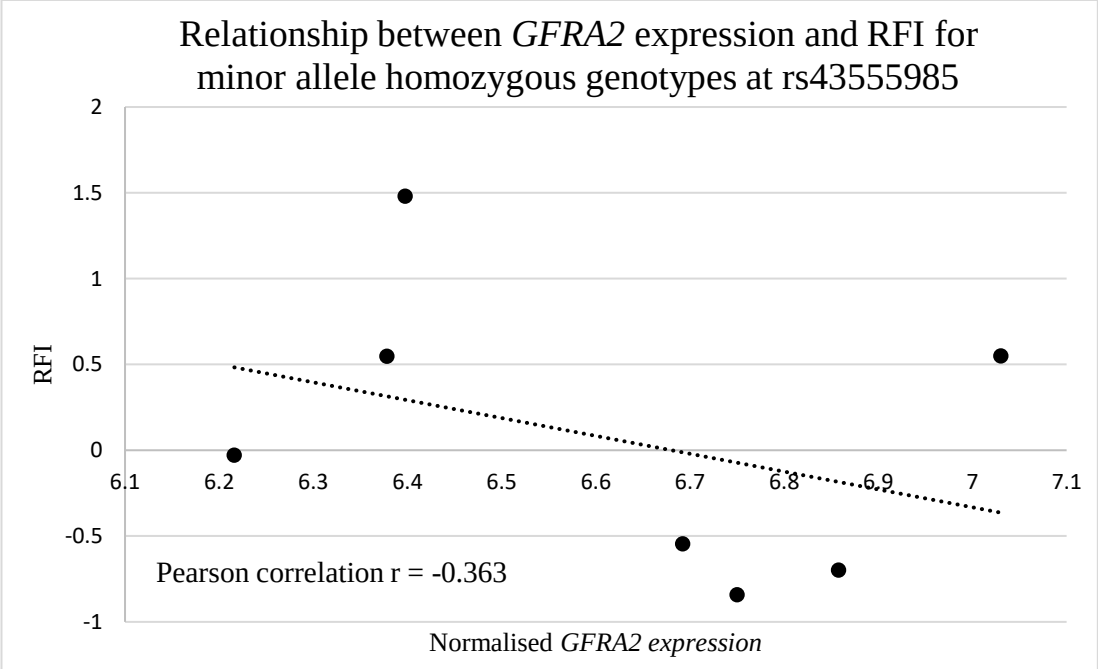
Supplementary Figure 2.1b.



Supplementary Figure 2.1c.



Supplementary Figure 2.1d



Supplementary Figure 2.1. Scatterplots illustrating the relationship between *GFRA2* expression and RFI for genotypes at rs43555985.

Supplementary Figure 2.1a illustrates the relationship between *GFRA2* expression and RFI for all genotypes of rs43555985. Supplementary Figure 2.1b illustrates the relationship between *GFRA2* expression and RFI for animals with major allele homozygous genotypes at rs43555985. Supplementary Figure 2.1c highlights the relationship between *GFRA2* expression and RFI for animals with heterozygous genotypes at rs43555985. Supplementary Figure 2.1d illustrates the relationship between *GFRA2* expression and RFI for animals with minor allele homozygous genotypes at rs43555985.

Chapter 3

The effect of breed and diet type on the global transcriptome of hepatic tissue in beef cattle divergent for feed efficiency

Preamble to Chapter 3: Statement of contribution

This chapter reports work investigating differential gene expression across two breeds of beef steer offered three varying diets throughout their lifetime. This animal model was created as part of a large experiment investigating the repeatability of RFI ranking across dietary stage, of which this investigation was part. The animal model was generated by Prof. David Kenny, Dr. Mark McGee, Dr. Claire Fitzsimons and Séan Coyle, who also collected the biopsies for this investigation. I carried out RNA extraction and RNA-Seq library preparation for this work. I also carried out bioinformatic analysis in conjunction with Dr. Gordon Blackshields. I wrote the first draft of the manuscript which comprises this Chapter, with edits and contributions from my supervisors Dr. Sinéad Waters and Dr. Derek Morris. All listed authors provided comments on the manuscript prior to submission. This manuscript is currently under review in BMC Genomics.

3. The effect of breed and diet type on the global transcriptome of hepatic tissue in beef cattle divergent for feed efficiency.

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3.1. Abstract

3.1.1. Background

Feed efficiency is an important economic and environmental trait in beef production, which can be measured in terms of residual feed intake (RFI). Cattle selected for low-RFI (feed efficient) have similar production levels but decreased feed intake, while also emitting less methane. RFI is difficult and expensive to measure and is not widely adopted in beef production systems. However, development of DNA-based biomarkers for RFI may facilitate its adoption in genomic-assisted breeding programmes. Cattle have been shown to re-rank in terms of RFI across diets and age, while also RFI varies by breed. Therefore, we used RNA-Seq technology to investigate the hepatic transcriptome of RFI-divergent Charolais (CH) and Holstein-Friesian (HF) steers across three dietary phases to identify genes and biological pathways associated with RFI regardless of diet or breed.

3.1.2. Results

Differential gene expression analysis was carried out between high- and low-RFI CH and HF steers following three dietary stages in order to identify genes associated with RFI across breed and diet. RFI was measured during a high-concentrate phase, a zero-grazed grass phase and a final high-concentrate phase. In total, 322 and 33 differentially expressed genes (DEGs) were identified across all diets for CH and HF steers, respectively. Three genes, *GADD45G*, *HP* and *MID1IP1*, were differentially expressed in CH when both the high-concentrate zero-grazed grass diet were offered. Two and ten canonical pathways were enriched across all diets for CH and HF steers, respectively. Eleven of these canonical pathways were related to immune function. Fourteen biological functions, including lipid metabolism, were enriched across all breed and diet combinations.

3.1.3. Conclusions

The absence of common differentially expressed genes across all dietary phases and breeds in this study supports previous reports of the re-ranking of animals in terms of RFI when offered differing diets over their lifetime. However, we have identified

several biological pathways and processes including the immune response and lipid metabolism as associated with RFI divergence independent of diet or breed, emphasising the previously reported roles of these biological processes with respect to RFI.

3.2. Introduction

Feed provision accounts for more than 70% of direct costs in beef production systems (Finneran et al., 2010). Selection of feed efficient cattle would improve profits by reducing expenditure on feed while maintaining output (Berry and Crowley, 2013). Moreover, there is increasing pressure on the global agri-food industry to improve its environmental footprint, while increasing output to meet the growing demand for protein (Foley et al., 2011). Selection for feed efficient cattle could maintain output while concurrently decreasing methane emissions, as it has been illustrated that low-RFI cattle emit less methane than their inefficient counterparts (Fitzsimons et al., 2013).

Feed efficiency has several methods of measurement including residual feed intake (RFI) (Archer et al., 1999), which is defined as the difference between an animal's actual and predicted feed intake. RFI has gained popularity as a measure of feed efficiency due to its moderate heritability and its phenotypic independence from production traits (Berry and Crowley, 2013). It has been suggested that variation in RFI may be due to differences in an animals physiological processes, such as those that occur in the liver (Cantalapiedra-Hijar et al., 2018). The liver is a major metabolic organ in ruminants, typically consuming 24% of total energy (Reynolds, 1992). The liver distributes nutrients to organs for both maintenance and production, amongst other functions such as gluconeogenesis (Lawrence et al., 2012b). The liver also plays a role in physiological processes such as the immune response, glucose metabolism and lipid metabolism (Nafikov and Beitz, 2007, Donkin, 2012). Due to the multifactorial role of the ruminant liver, any variation in its gene expression may reflect divergent efficiency of overall metabolic and physiological function potentially leading to phenotypic differences in RFI.

Incorporating RFI into breeding programmes would enable selection of feed efficient cattle, thereby improving farm profits. However, RFI or indeed any measure of feed efficiency is not widely included in breeding programmes worldwide due to the expense associated with its measurement. The calculation of RFI requires an expensive and often labour intensive performance measurement period during which individual feed intake and weight gain are recorded for each animal (Nielsen et al., 2013). RFI's observed heritability, with an estimated range of 0.26-0.54 (Berry and Crowley, 2013), has led to considerable international interest in the discovery of accurate and robust biological markers of RFI. The identification of such biomarkers would circumvent the necessity for direct measurement of the trait and would enable incorporation of this trait into genomic selection assisted breeding programmes (Cole and VanRaden, 2017).

To facilitate genomic assisted selection of feed efficient cattle, robust genetic markers must be identified. These biomarkers must be repeatable as differences in breed (Crowley et al., 2010, Schenkel et al., 2004) and physiological age (Kelly et al., 2010b, Coyle et al., 2016), as well as genotype-by-environment interactions, have been observed to cause re-ranking of cattle for RFI status (Kenny et al., 2018, Durunna et al., 2011b). This represents a challenge in identifying biomarkers for RFI applicable to beef production systems, as beef cattle are primarily raised on grass based diets, followed by a finishing period during which they are typically offered an energy dense-concentrate diet (Mulliniks et al., 2015). Additionally, biomarkers identified for selection of feed efficient beef cattle must be applicable to the multiplicity of breeds and crossbred cattle typically found within the global beef production industry (Rolf et al., 2014, Wickham et al., 2012). Therefore, it is important that genetic variants included in genomic breeding programmes are robust across dietary phases and physiological age, regardless of breed.

To identify genetic variants associated with RFI, genome-wide association studies (GWAS) have been conducted which have identified single nucleotide polymorphisms (SNPs) and quantitative trait loci (QTL) associated with RFI (Seabury et al., 2017, Saatchi et al., 2014a, Abo-Ismail et al., 2014, Rolf et al., 2012,

Higgins et al., 2018). However, there is little overlap in associated QTL regions and SNPs identified in these studies. Another method to identify SNPs of interest is using a candidate gene approach (Tabor et al., 2002), whereby genes associated with RFI-divergence may be sequenced and interrogated for SNPs to be included in breeding programmes. Candidate genes suitable for sequencing can be uncovered by identifying differentially expressed genes (DEGs) for the trait of interest. RNA-Seq, a method by which all expressed genes within a tissue are profiled (Wang et al., 2009), has been used to identify DEGs associated with RFI. RNA-Seq offers several advantages over other transcriptome profiling methods including that the technology facilitates the entire transcriptome of an organism to be investigated rather than known genes as is the case for microarray analysis or real time PCR (Wang et al., 2009).

RNA-Seq analyses have been conducted to investigate variation in gene expression between RFI-divergent cattle in several tissues including liver (Tizioto et al., 2015, Alexandre et al., 2015), skeletal muscle (Tizioto et al., 2016) and rumen epithelial tissue (Kong et al., 2016b). Recently, Mukiibi et al., (2018) observed five DEGs across three breeds of Canadian cattle offered the same diet. No published studies have identified genes consistently differentially expressed across several dietary phases in multiple breeds of cattle.

To identify genes associated with RFI across breed and diet, we conducted RNA-Seq analysis of the liver transcriptome of two breeds of cattle subjected to three dietary regimens: a high-concentrate diet, a zero-grazed grass diet and cattle were finished on a high-concentrate diet. The aims of this study were: (i) to elucidate the underlying biology of RFI by investigating key genes and pathways implicated in RFI divergence and (ii) to identify genes and biological functions associated with RFI across multiple breeds and dietary phases in order to highlight candidate genes for further interrogation as potential biomarkers for RFI.

3.3. Materials and methods

3.3.1. Animal model

All procedures involving animals in this study were reviewed and approved by the Teagasc animal ethics committee and were conducted under an experimental licence issued by the Health Products Regulatory Authority (AE19132/P029), in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendment of Cruelty to Animals Act 1876) Regulations 2002 and 2005.

This experiment was conducted as part of a larger study examining genotype-by-environment interactions for and repeatability of feed efficiency across growing and finishing stages of beef production, during which diets offered differed in energy density and chemical composition. The animal model used was described in detail previously (Coyle et al., 2016, Coyle et al., 2017).

Briefly, 90 Charolais (CH) and 77 Holstein-Friesian (HF) steers were offered different diets throughout their lifespan. Cattle were first offered a high-concentrate diet (H1) in the growing phase, followed by a zero-grazed grass (ZG) diet during the growing phase and then were returned to a high-concentrate diet (H2) during the finishing phase. Individual dry matter intake (DMI) and growth were measured over these three individual feeding phases and RFI was calculated at the end of each of these stages. Prior to each of these RFI measurement trials, a 21-day dietary adaptation phase was conducted. At the start of the first dietary phase (H1) the mean age (standard deviation) of the steers was 283 days (18.3) and 306 days (7.7), for CH and HF, respectively (Figure 3.1). During each individual feeding phase the health of all cattle was monitored. Any animal which required treatment was noted and excluded from downstream analysis.

During H1 and H2, steers were individually offered the same high-concentrate diet ad libitum and a restricted allowance of grass silage daily in order to maintain healthy rumen function. The high-concentrate diet consisted of 860g/kg rolled barley, 60g/kg soya bean meal, 60g/kg molasses and 20g/kg minerals and vitamins.

During the ZG phase, steers were individually offered ad libitum zero-grazed grass (DM 183g/kg). Grass was harvested twice daily from *Lolium perenne* dominant swards using a zero-grazer. Cattle were given unrestricted access to fresh, clean drinking water throughout all phases of this study.

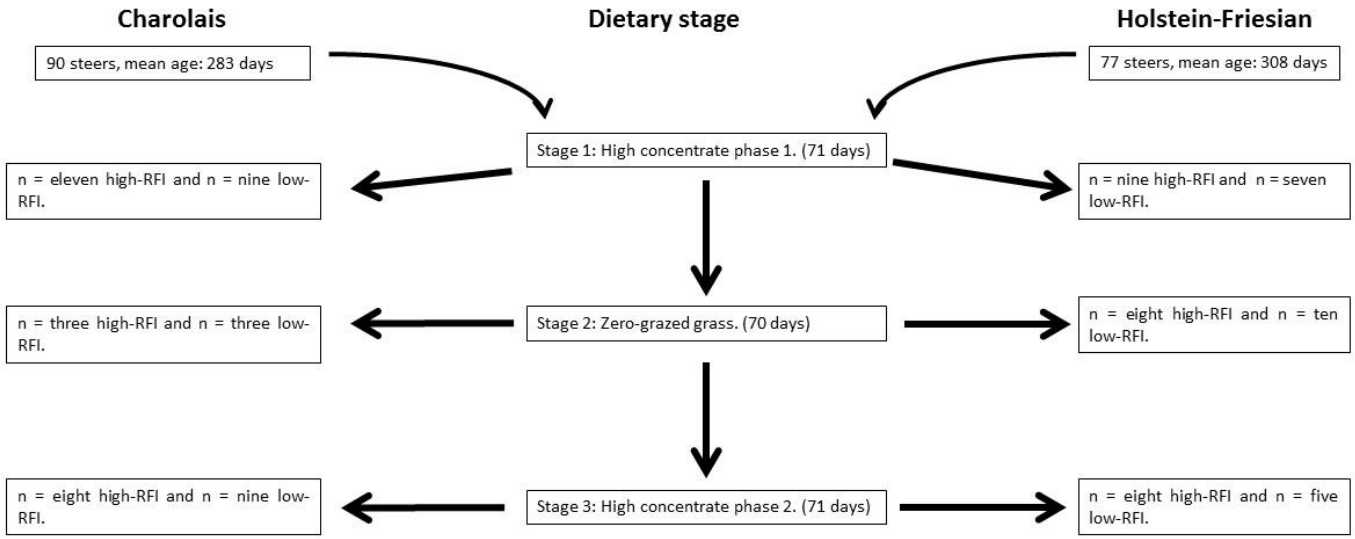

Figure 3.1. Outline of the feeding trial design during which RFI was measured. During each dietary stage, steers were offered the respective diet for 90 days following a period of dietary adaptation. At the end of each dietary stage, liver biopsies were taken and RFI was calculated. Within breed, all steers were ranked for RFI. Biopsies from the most divergent (n = 12 high and n = 12 low) steers were taken for RNA-Seq library preparation.

3.3.2. Computation of traits

At the end of each dietary phase, average daily gain (ADG) of individual steers was calculated as the coefficient of the linear regression of BW (kg) on time (days) using the GLM procedure of SAS 9.3 (SAS Inst. INC., Cary, NC, USA). Mid-test metabolic weight (MBW) was computed as $BW^{0.75}$ halfway through each test period, which was estimated from the intercept and the slope of the regression line through all $BW^{0.75}$ observations.

Predicted DMI was computed for each steer, within breed, by regressing DMI on MBW and ADG using a multiple regression model. The model used to compute predicted DMI was:

$$Y_j = \beta_0 + \beta_1 MBW_j + \beta_2 ADG_j + e_j,$$

where Y_j was the average DMI of the *j*th steer, β_0 is the regression intercept, β_1 is the partial regression coefficient on MBW, β_2 is the partial regression coefficient on ADG and e_j is the random error associated with the *j*th animal. RFI was calculated as the difference between actual and predicted DMI. Steers were ranked by RFI within breed for each dietary phase, and the twelve most efficient (low RFI) and the twelve least efficient (high RFI) animals were identified for each breed and phase.

3.3.3. Sample collection, RNA extraction and cDNA library synthesis

Liver tissue was collected from all animals at the end of each dietary phase by percutaneous punch as described by McCarthy et al. (2009). Animals received local anaesthetic (5ml Adrenacaine, Norbrook Laboratories, Ireland Ltd.) and care was taken to ensure samples were consistently harvested from the same location for each animal. All instruments used for biopsy collection were sterilized, washed with 70% ethanol and treated with RNaseZap (Ambion, Applera Ireland, Dublin, Ireland). All samples were washed in sterile DPBS, snap frozen in liquid nitrogen and stored at -80°C prior to further analysis.

50 mg of the biopsied tissue was used for the isolation of total RNA. Samples were homogenised using a rotor-strator tissue lyser (Qiagen, UK) in 3ml of QIAzol (Qiagen, UK). RNA was extracted and purified using the RNeasy plus Universal kit (Qiagen, UK) as per the manufacturer's instructions. RNA quantity was determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Quality control checks were carried out on isolated RNA using the RNA 6000 RNA Nano Lab Chip Kit and the Agilent Bioanalyser 2100 (Agilent Technologies Ireland Ltd., Dublin, Ireland). Samples displaying a RNA integrity number of greater than 8 were deemed of sufficient quality for analysis, and were subjected to cDNA synthesis.

cDNA libraries were prepared for sequencing using the Illumina TruSeq stranded mRNA sample prep kit (Illumina, San Diego, CA, USA) as per manufacturer's instructions. Library validation was conducted using the DNA 1000 Nano Lab Chip which was read using the Agilent Bioanalyser 2100 (Agilent Technologies Ltd. Dublin, Ireland). Library concentration was assessed using a Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Samples with a DNA concentration of greater than 25ng/µl were subjected to further analysis. Libraries were pooled and 50 base-pair, single-end sequencing was conducted using an Illumina HiSeq 2500. Prior to library generation, some samples were excluded due to poor RNA quality. A total of 45 CH and 58 HF libraries were sequenced successfully (Figure 3.1). All sequence data generated as part of this study has been submitted to the Gene Expression Omnibus repository and can be accessed using the accession number GSE111464.

3.3.4. RNA-Seq data analysis

Sequencing data were supplied in FASTQ format. Adapter and low quality sequence data were removed using cutadapt (v. 1.13) (Martin, 2011). Reads were retained if they had an average base quality of at least 30 and a minimum length of 20bp. FastQC (v. 0.11.5) (Andrews, 2010) was used for quality assessment of the filtered data. Both cutadapt and FastQC were called using TrimGalore! (v.0.4.3) (Krueger,

2015). After trimming, libraries with less than 10^7 reads were discarded. Before filtering an average of 29.05 million reads per sample were generated, and these reads had an average GC-content of 47.99% with 96.92% of bases having a quality score greater than 30. Following filtering, average reads per sample remained at 29.05 million, and average GC-content increased to 48.06%. Post-filtering, 99.05% of bases had a Q score greater than 30.

Reads were mapped to the bovine reference genome (UMD3.1) using STAR (v.2.5.1) (Dobin et al., 2013). Protein coding genes were supplied from the Ensembl (Zerbino et al., 2018) version 87 annotation of the *Bos taurus* genome (Zimin et al., 2009). The STAR parameter "*quantMode GeneCounts"* was used to quantify the mapped reads at the gene level.

Analysis of the gene count data was carried out using the Bioconductor (Gentleman et al., 2004) package DESeq2 (Love et al., 2014) (v. 1.16.1). Raw gene counts were provided to DESeq2 and an analysis pipeline, DESeq, was called from the DESeq2 R package. This pipeline was used to accurately calculate dataset-specific analysis parameters and apply negative binomial GLM fitting to the data for use in the subsequent differential expression analysis. Any samples identified as outliers were removed. For each pair of experimental groups under investigation, a list of differentially expressed genes (DEGs) was extracted directly from the DESeq data. A Benjamini-Hochberg correction was applied to account for multiple test burden (Benjamini and Hochberg, 1995). Following correction, an adjusted *P*-value of <0.1, the recommended threshold for DESeq2, was used to denote significance.

3.3.5. Pathway and functional enrichment analysis

Each list of DEGs was further investigated using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA). DEGs, along with their respective fold-changes and adjusted *P*-values were submitted to IPA for analysis. IPA allows examination of over-represented biological pathways and biological functions (Keogh et al., 2016b). IPA core analysis was performed on genes identified as

statistically significant (adjusted $P < 0.1$) following DESeq2 analysis. However, if too few genes reached an adjusted *P*-value < 0.1, genes that reached a nominal significance of *P*-value < 0.05 were included for analysis. Consequently, 160, 158 and 515 genes were uploaded to IPA for the CH H1, ZG and H2 diets, respectively, while 987, 368 and 577 genes were uploaded to IPA for the HF H1, ZG and H2 diets, respectively.

Genes were then mapped to IPA biological functions and canonical pathways. Biological functions and canonical pathways were considered to be significantly enriched if the *P*-value of the overlap between the input gene list and the genes within the database for a given function or pathway was less than 0.05. Upregulation or downregulation of functions or pathways was determined by a Z-score, as calculated by IPA from the expression levels of input genes in a function or pathway. A negative Z-score represented downregulation of a function or pathway, while a positive Z-score represented upregulation.

3.4. Results

3.4.1. Animal model

Across all three dietary phases and within breed, high RFI steers consumed more feed on average than their low RFI counterparts ($P < 0.001$), while having a similar ADG ($P > 0.05$). As expected, within breed and dietary phase no statistically significant difference in MBW and ADG was observed between the two RFI groups (Table 3.1). No statistical difference in MBW and ADG was also observed for the animals for which RNA-Seq libraries were generated (Table 3.2) however, the high-RFI CH steers offered the ZG diet displayed a trend to consume less feed than their low RFI counterparts ($P = 0.07$).

Table 3.1. Feed intake, RFI and growth traits for the entire population of low- and high-RFI steers during different dietary phases.

Trait	Diet-Breed	Low	High	*P*-value
DMI (kg/d)	H1.CH	7.8	9	<0.001
	H1.HF	8.3	9.3	<0.001
	ZG.CH	8.8	9.4	<0.001
	ZG.HF	9.1	10	<0.001
	H2.CH	10.8	12.3	<0.001
	H2.HF	11.6	13.6	<0.001
RFI (kg DM/d)	H1.CH	-0.5	0.56	<0.001
	H1.HF	-0.5	0.53	<0.001
	H2.CH	-0.75	0.76	<0.001
	H2.HF	-1.01	1.03	<0.001
	ZG.CH	-0.35	0.35	<0.001
	ZG.HF	-0.42	0.56	<0.001
ADG (kg)	H1.CH	1.3	1.4	0.43
	H1.HF	1.4	1.4	0.78
	H2.CH	1.4	1.4	0.64
	H2.HF	1.3	1.3	0.98
	ZG.CH	1.4	1.4	0.96
	ZG.HF	1.2	1.3	0.85
MBW (kg)	H1.CH	95	96	0.79
	H1.HF	81	80	0.90
	H2.CH	96	97	0.79
	H2.HF	82	81	0.90
	ZG.CH	96	97	0.8
	ZG.HF	82	80	0.9

DMI = dry matter intake; RFI = residual feed intake; ADG = average daily gain; MBW = metabolic body weight; CH = Charolais; HF = Holstein-Friesian; Low = low RFI; High = high RFI; H1 = high concentrate diet 1; H2 = high concentrate diet 2; ZG = zero-grazed grass diet.

Table 3.2. Feed intake, RFI and growth traits for the low-and high-RFI steers for which RNA-Seq libraries were successfully generated during different dietary phases.

Trait	Diet-Breed	Low	High	*P*-value
DMI (kg/d)	H1.CH	7.5 (n=9)	9.1 (n=11)	<0.001
	H1.HF	7.7 (n=7)	9.7 (n=9)	<0.001
	ZG.CH	8.4 (n=3)	9.7 (n=3)	0.07
	ZG.HF	8.8 (n=10)	10.3 (n=8)	<0.001
	H2.CH	10.5 (n=9)	12.9 (n=8)	<0.001
	H2.HF	10.8 (n=5)	13.6 (n=8)	0.002
RFI (kg DM/d)	H1.CH	-0.8	0.8	<0.001
	H1.HF	-0.9	0.8	<0.001
	ZG.CH	-0.5	0.5	<0.001
	ZG.HF	-0.7	0.7	<0.001
	H2.CH	-1.1	1.2	<0.001
	H2.HF	-1.9	1.3	<0.001
ADG (kg)	H1.CH	1.3	1.3	0.9
	H1.HF	1.4	1.2	0.2
	ZG.CH	1.3	1.3	0.6
	ZG.HF	1.3	1.2	0.2
	H2.CH	1.4	1.4	0.8
	H2.HF	1.3	1.4	0.7
MBW (kg)	H1.CH	95	95	0.76
	H1.HF	81	80	0.59
	ZG.CH	113	118	0.53
	ZG.HF	105	104	0.55
	H2.CH	137	142	0.45
	H2.HF	130	127	0.56

DMI = dry matter intake; RFI = residual feed intake; ADG = average daily gain; MBW = metabolic body weight; CH = Charolais; HF = Holstein-Friesian; Low = low RFI; High = high RFI; H1 = high concentrate diet 1; H2 = high concentrate diet 2; ZG = zero-grazed grass diet.

3.4.2. Differential gene expression analysis

A total of 160, 158 and 4 genes (adjusted $P < 0.1$) were identified as differentially expressed between high and low RFI CH cattle for the H1, ZG and H2 diets, respectively. For HF; 26, 2 and 5 (adjusted $P < 0.1$) were differentially expressed between RFI cohorts for H1, ZG and H2, respectively. The top DEGs for each comparison are represented in Tables 3.3 and 3.4 for CH and HF, respectively. All DEGs for each breed and diet are listed in Supplementary Table 3.1.

In CH cattle, three DEGs were common to the H1 and ZG diets, while no gene was common to all three diets. These genes shared between H1 and ZG in CH were *growth arrest and DNA damage inducible gamma* (*GADD45G), haptoglobin precursor* (*HP*) and *MID1 interacting protein 1* (*MID1IP1*). *HP* was upregulated in low RFI steers across both diets, while *MID1IP1* was downregulated in the same diets. However, relative to high RFI, *GADD45G* was upregulated in low RFI CH steers offered the H1 diet, while it was downregulated in low RFI steers offered the ZG diet. There were no common DEGs across dietary phases for HF cattle. Similarly, no DEG was shared across breeds, for any of the three dietary phases.

Table 3.3. The most significantly differentially expressed genes between high and low RFI Charolais steers across three dietary phases as determined by *P*-value.

Diet and Breed	Gene	LogFC	*P*-value
CH.H1	*TNFAIP3*	0.66	0.0002
	KRBA1	1.39	0.00069
	SIK1	1.33	0.0043
	IRS2	1.23	0.0043
CH.ZG	*SLC39A4*	-2.68	4.58E-09
	BHMT2	1.26	7.60E-08
	TNC	2.02	2.95E-07
	ENSBTAG00000016032	1.58	2.95E-07
	ABCA6	1.16	0.0001
CH.H2	*LOC768255*	-3.55	0.00024
	GIMAP4	-3.08	0.0065

RFI = residual feed intake; CH = Charolais; H1 = high concentrate diet 1; H2 = high concentrate diet 2; ZG = zero-grazed grass diet; LogFC = log_2fold-change in low-RFI steers compared to high-RFI steers; *P*-value = Benjamini-Hochberg corrected *P*-value to account for multiple testing.

Table 3.4. The most significantly differentially expressed genes between high- and low-RFI Holstein-Friesian steers across three dietary phases, as determined by *P*-value.

Diet and breed	Gene	LogFC	*P*-value
HF.H1	*SNRPD3*	-0.37	0.0010
	AK3	-0.35	0.0013
	GSTM1	-0.87	0.0061
	MOB3B	0.72	0.0061
	LOC782233	-5.45	0.0061
	HPRT1	-0.33	0.011
	ENSBTAG00000032859	-0.66	0.016
	ACMSD	-1.04	0.024
	PARM1	1.15	0.024
	ANPEP	1.10	0.024
	GUCY2D	-0.89	0.024
	GSTA4	-0.88	0.026
	RAB4A	-0.29	0.026
	CYTH3	0.52	0.026
	HSD17B6	-0.36	0.028
	RAC1	0.35	0.038
HF.ZG	*INPP1*	0.73	0.005
	ALAS1	-0.94	0.074
HF.H2	*UOX*	0.85	0.028
	C1R	7.45	0.055
	LOC100295234	2.92	0.055
	SNCA	-3.61	0.055
	FBP2	-3.06	0.055

RFI = residual feed intake; HF = Holstein-Friesian; H1 = high concentrate diet 1; H2 = high concentrate diet 2; ZG = zero-grazed grass diet; LogFC = $\log_2$fold-change in low-RFI steers compared to high-RFI steers; *P*-value = Benjamini-Hochberg corrected *P*-value to account for multiple testing.

3.4.3. Pathway analysis and functional enrichment

For the CH cohort 141, 143 and 422 genes mapped to the IPA knowledge database for the H1, ZG and H2, respectively. The transcriptome of the CH steers offered the H2 diet contained too few DEGs for IPA therefore nominally significant genes were mapped to IPA for the H2 diet. For the HF steers 863, 307 and 489 nominally significant genes mapped to IPA for H1, ZG and H2, respectively.

Following IPA analysis, 103, 77 and 37 significantly enriched (Fisher's exact *P*-value < 0.05) canonical pathways were identified for H1, ZG and H2, respectively, in the CH cohort. Table 3.5 illustrates the top ten canonical pathways affected by RFI divergence between CH steers for the H1, ZG and H2 diets. For the HF steers 74, 65 and 143 significantly enriched canonical pathways were identified for the H1, ZG and H2 diets, respectively. Table 3.6 lists the top ten canonical pathways for the HF steers, while all enriched canonical pathways are listed in Supplementary Table 3.2. A total of two pathways were enriched across all three diets for CH, while ten canonical pathways were enriched across all three dietary stages within the HF breed (Table 3.7). The two pathways for the CH cohort were interlukin-6 (IL-6) signalling and acute phase response signalling. Similarly, nine of the ten canonical pathways detected in the HF cohort are related to immunological signalling or autoimmune disease.

Following IPA analysis, 14 enriched (Fisher's exact *P*-value < 0.05) biological functions were significant across all dietary phases for both low-RFI CH and HF (Supplementary Table 3.3).

Table 3.5. The top ten canonical pathways for CH steers within each dietary phase.

Diet-Breed Combination	Canoncial pathway	Differentially Expressed Genes	*P*-value
CH.H1	Toll-like Receptor Signalling	*IL1A, JUN,* ***MAP2K6****, NFKBIA, TNFAIP3,* ***UBA52***	8.71E-09
	CD40 Signalling	*IRS2, JUN,* ***MAP2K6****, NFKBIA, TNFAIP3*	0.00015
	IL-6 Signalling	**CSNK2B**, IL1A, IRS, JUN, **MAP2K6**, NFKBIA	0.00017
	Aryl Hydrocarbon Signalling	**ALDH9A1**, IL1A, JUN, MYC, TFDP1, **TGM2**	0.00028
	Cholecystokinin/Gastrin mediated Signalling	IL1A, JUN, **MAP2K6**, *MAPK7,* ***RND3***	0.00046
	p53 Signalling	*GADD45G, IRS2, JUN, TNFRSF10A, TP53INP1*	0.00071
	TNFR2 Signalling	*JUN, NFKBIA, TNFAIP3*	0.00093
	Acute Phase Response Signalling	*HP, IL1A, JUN,* ***MAP2K6****, NFKBIA,* ***SAA1***	0.00097
	IL-10 Signalling	*IL1A, JUN,* ***MAP2K6****, NFKBIA*	0.001
	NFKB Signalling	***CSNK2B****, IL1A, IRS2,* ***MAP2K6****, NFKBIA, TNFAIP3*	0.0013
CH.ZG	Glycine Betaine Degradation	*BHMT2, DMGDH,* ***SARDH***	0.000029
	Acute Phase Response Signalling	*C5, FGG, HP,* ***HRAS****, LBP, SERPINA3*	0.0009
	Hereditary Breast Cancer Signalling	***CCND1, FGFR3, GADD45G, HDAC5, HRAS***	0.0027
	EIF2 Signalling	***ATF5, CCND1, EIF1, FGFR3, FGFR3, HRAS, RPL13***	0.0033
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	*C5,* ***CCND1, FGFR3, HRAS, IL17RC, MIF, TRAF4***	0.0043
	Extrinsic Prothombin Activation Pathway	*F5, FGG*	0.0045
	Chronic Myeloid Leukemia Signalling	***CCND1, FGFR3, HDAC5, HRAS***	0.0056
	Germ Cell-Sertoli Junction Signalling	***BCAR1, FGFR3, HRAS, TUBA4A, TUBB4B***	0.0057
	Methylglyoxal Degradation VI	***LDHD***	0.0063
	GADD45 Signalling	***CCND1, GADD45G***	0.0063
CH.H2	IL-10 Signalling	***ARG2****, CD14,* ***FCGR2B****, IL1RAP, IL1RL2,* ***MAP2K6***	0.0019

	Circadian Rhythm Signalling	*ATF4, CLOCK,* ***CREB3L4****,* ***NR1D1***	0.0038

Diet-Breed Combination	**Canoncial pathway**	**Differentially Expressed Genes**	***P*-value**
	Sirtuin Signalling Pathway	*ACADL,* ***ARG2****, CDH1, CLOCK,* ***CXCL8****,* ***LDHB****, MT-ND1, MT-ND5, MT-ND6,* ***NDUFB1****,* ***OGG1****, PPARGC1A,* ***ZBTB14***	0.0041
	IL-6 Signalling	*CD14, CSNK2A2,* ***CXCL8****,IL1RAP, IL1RL2,* ***MAP2K6****, PIK3R1,* ***PIK3R6***	0.0041
	The Visual Cycle	***AKR1C3,LRAT, RBP5***	0.0061
	Clathrin-mediated Endocytosis Signalling	*ACTA2, ALB, AP1G1, CBL, CSNK2A2, FGF21, MYO1E, PIK3R1,* ***PIK3R6****, PPP3CC*	0.0064
	Osteoarthritis Pathway	*ATF4,* ***CREB3L4****,* ***CXCL8****,* ***ELF3****,* ***FZD3****, IL1RL2, IL1RAP,* ***P2RX7****, PPARGC1A,* ***SPP1***	0.0075
	FGF Signalling	*ATF4,* ***CREB3L4****, FGF21,* ***MAP2K6****, PIK3R1,* ***PIK3R6***	0.0078
	LXR/RXR Activation	*ALB,* ***ARG2****, CD14, GC, IL1RAP, IL1RL2, PON3*	0.0084
	PPARa/RXRa Activation	*ACADK, CLOCK, CYP2C18, CYP2C18, CYP2C23, IL1RAP, IL1RL2,* ***MAP2K6, NR2C2****, PPARGC1A*	0.0094

H1 = high concentrate, phase 1; H2 = high concentrate, phase 2; ZG = Zero-grazed grass; CH = Charolais; *P*-value = Fisher's exact test *P*-value, bold text indicates gene downregulation in low-RFI steers.

Table 3.6. The top ten canonical pathways for HF steers within each dietary phase.

Diet-Breed Combination	Canonical pathway	Differentially Expressed Genes	*P*-value
HF.H1	Glutathione-mediated Detoxification	*ANPEP*, ***GGH***, ***GSTA1***, ***GSTA4***, ***GSTM1***, ***GSTM4***, ***GSTT3***	0.00015
	Fatty Acid Beta-oxidation I	***ACAA2***, *ACSL1*, ***ECHS1***, ***IVD***, ***SLC27A2***, ***SLC27A4***,***SLC27A6***	0.00019
	Uracil Degradation II (Reductive)	***DPYD***, ***DPYS***, ***UPB1***	0.00023
	Thymine Degradation	***DPYD, DPYS, UPB1***	0.0023
	Stearate Biosynthesis I (Animals)	***ACOT4***, *ACOT9*, *ACSL1*, ***FASN***, ***PORCN***, ***SLC27A2***, ***SLC27A4***, ***SLC27A6***	0.00026
	Colorectal Cancer Metastisis Signalling	***APC***, *ARRB1*, ***CASP3***, *CDH1*, *E2F4*, ***FGFR3***, *FOS*, *GNAS*, ***GNG10***, *GRK2*, *LRP6*, *PTGER4*, *RAC1*, *RAP2A*, *RELA*, *RHOF*, ***RHOU***, ***SIAH1***, *TGFB1*, *TLR7*, *TNFRSF1A*, *TP53*	0.004
	2-Oxobutanoate Degradation I	***DLD***, ***MUT***, ***PCCA***	0.00055
	Crosstalk between Dendritic Cells and Natural Killer Cells	*CCR7*, *CD40*, *CD40LG*, *HLA-DRA*, *ICAM3*, *IL2RG*, *LTB*, *PRF1*, *RELA*, *TLR7*, *TREM2*	0.00066
	Molecular Mechanisms of Cancer	***APC***, *ARHGEF3*, *ARHGEF5*, ***ARHGEF19***, ***CASP3***, *CCND3*, *CDC25B*, *CDH1*, *CDK20*, *DAXX*, *E2F4*, ***FGFR3***, *FOS*, *FYN*, *GNA13*, ***GNA14***, *GNAS*, *LRP6*, *MAX*, *PRKD3*, *RAC1*, *RAP2A*, *RAPGEF1*, *RASGRP1*, *RELA*, *RHOF*, ***RHOU***, *TGFB1*, *TP53*	0.008
	PXR/RXR Activiation	***ALDH1A1***, ***CYP1A2***, ***G6PC***, ***GSTA1***, ***GSTM1***, ***PCK2***, *RELA*, ***SLC01B3***, ***SULT2A1***	0.00088
HF.ZG	LXR/RXR Activation	*ABCG8*, *APOC3*, ***ARG2***, ***CYP7A1***, *IL1R2*, ***LY96***,	1.10E-07

		MYLIP, ***NOS2***, ***PTGS2***, *SAA1*, ***TNF***, *TTR*	
Diet-Breed Combination	**Canonical pathway**	**Differentially Expressed Genes**	***P*-value**
	Superpathway of Citrulline Metabolism	***ALDH18A1***, ***ARG1***, ***ARG2***, ***NOS2***	4.21E-05
	Pathogenesis of Multiple Sclerosis	***CXCL9***, ***CXCL10***, ***CXCL11***	2.02E-04
	Citrulline Biosynthesis	***ALDH18A1***, ***ARG1***, ***ARG2***, ***NOS2***	2.02E-04
	PXR/RXR Activation	***ALAS***, *CES2*, ***CYP7A1***, *IGFBP1*, ***SULT2A1***, ***TNF***	2.65E-04
	Xenobiotic Metabolism Signalling	***ALDH18A1***, ***ALDH7A1***, *CES2*, *CES5A*, *GCLC*, ***GRB2***, *MAP3K15*, ***NOS2***, *PPM1J*, ***SULT2A1***, ***TNF***, ***UGT8***	8.49E-04
	Type I Diabetes Mellitus Signalling	***CPE***, ***HLA-DMA***, ***HLA-DQA1***, ***HLA-DRA***, ***ICA1***, ***NOS2***, ***TNF***	8.57E-04
	LPS/IL-1 Mediated Inhibition of RXR Function	*ABCG8*, ***ALAS1***, ***ALDH18A1***, ***ALDH7A1***, *CES2*, ***CYP7A1***, *IL1R2*, ***LY96***, ***SULT2A1***, ***TNF***	1.03E-03
	Arginine Degradation I (Arginase Pathway)	***ARG1***, ***ARG2***	1.10E-03
HF.H2	Th1 and Th2 Activation Pathway	***CCR1***, ***CCR5***, ***CCR8***, ***CD40***, ***CD274***, ***CD3D***, ***CD3E***, ***CXCR3***, ***HLA-B***, ***HLA-DMA***, ***HLA-DOM***, ***HLA-DRA***, *IL18*, *IL10RB*, ***NFKB1***, ***PRKCQ***, ***TIMD4***	1.79E-07
	Th1 Pathway	***CCR5***, ***CD40***, ***CD274***, ***CD3D***, ***CD3E***, ***CXCR3***, ***HLA-B***, ***HLA-DMA***, ***HLA-DOA***, ***HLA-DRA***, *IL18*, *IL10RB*, ***IL12RB1***, ***NFKB1***, ***PRKCQ***	3.42E-07
	Pathogenesis of Multiple Sclerosis	***CCR1***, ***CCR5***, ***CXCL9***, ***CXCL10***, ***CXCR3***	6.21E-07
	iCOS-iCOSL Signalling in T-helper Cells	***CD40***, ***CD3D***, ***CD3E***, ***FCER1G***, ***HLA-B***, ***HLA-DMA***, ***HLA-DOA***, ***HLA-DRA***, ***LCP2***, ***NFKB1***, ***NFKBID***, ***PRKCQ***, ***RAC1***, ***ZAP70***	6.25E-07

	Interferon Signalling	***IF16***, ***IFIT1***, ***IFIT3***, ***IRF9***, ***ISG15***, ***MX1***, ***OAS1***, ***TAP1***	9.79E-07

Diet-Breed Combination	**Canonical pathway**	**Differentially Expressed Genes**	***P*-value**
	CD28 Signalling in T-helper Cells	***CD3D***, ***CD3E***, ***FCER1G***, ***FYN***, ***HLA-B***, ***HLA-DMA***, ***HLA-DOA***, ***HLA-DRA***,***LCP2***, ***NFKB1***, ***PRKCQ***, ***RAC1***, ***ZAP70***	1.48E-06
	Calcium Induced T-Lymphocyte Apoptosis	***ATP2A1***, ***CD3D***, ***CD3E***, ***FCER1G***, ***HLA-B***, ***HLA-DMA***, ***HLA-DOA***, ***HLA-DRA***, ***PRKCQ***, ***ZAP70***	1.85E-06
	ILK Signalling	***ACTN2***, ***ACTN3***, *DIRAS3*, ***FLNC***, ***LEF1***, ***MYC***, ***MYH2***, *MYH11*, ***MYL1***, ***NFKB1***, ***NOS2***, *PPP2CB*, *PPP2R2B*, *PPP2R5A*, ***RHOF***, *RHOU*, ***TNF***	2.10E-06

H1 = high concentrate, phase 1; H2 = high concentrate, phase 2; ZG = Zero-grazed grass; HF = Holstein-Friesian; *P*-value = Fisher's exact test *P*-value, bold text indicates gene downregulation in low-RFI steers.

Table 3.7. The canonical pathways shared across all diets for Charolais and Holstein-Friesian steers divergent in RFI

Breed	Canonical pathway	H1 *P*-value	H2 *P*-value	ZG *P*-value
CH	IL-6 Signalling	0.00017	0.0029	0.0085
	Acute phase response signalling	0.00076	0.042	0.00071
HF	Role of NFAT in regulation of the immune response	0.0071	0.000066	0.042
	T-Helper cell differentiation	0.0079	0.0000043	0.017
	Autoimmune thyroid disease signalling	0.011	0.00062	0.0041
	Th1 and Th2 activation pathway	0.015	0.00000015	0.013
	IL-17A signalling in gastric cells	0.016	0.017	0.0046
	Altered T- and B-Cell signalling in Rheumatoid Arthritis	0.026	0.000028	0.035
	Type I diabetes mellitus signalling	0.032	0.0000060	0.00081
	Th1 pathway	0.041	0.00000030	0.037
	Calcium-induced T-lymphocyte apoptosis	0.046	0.038	0.011
	Pathogenesis of Multiple Sclerosis	0.047	0.00000059	0.00019

CH: Charolais; HF: Holstein-Friesian; H1: High-concentrate, phase 1; H2: High-concentrate, phase 2; ZG: Zero-grazed grass.

3.5. Discussion

In order to identify genes associated with RFI status which are not subject to environmental influences, RNA-Seq was carried out on the liver transcriptome of CH and HF steers divergent for RFI across three dietary phases. This analysis identified two biological pathways significantly enriched across all dietary phases for CH steers, and ten biological pathways enriched across all dietary phases for HF steers. The majority of these pathways are related to immune function. At the individual gene level, three DEGs were found to be common to two diets within the CH breed. Genes implicated in processes previously associated with variation in RFI such as oxidative phosphorylation and extracellular matrix organisation were also identified as differentially expressed in this study (Chen et al., 2011, Kong et al., 2016b). The absence of consistently differentially expressed genes within RFI groups across dietary phase and breed supports the previously observed re-ranking of cattle when offered different diets (Coyle et al., 2016, Coyle et al., 2017).

3.5.1. Immune Function

Eleven of the twelve biological pathways associated with RFI in the present study were related to immunity. For the CH steers, the IL-6 signalling pathway was significantly enriched across all diets. This pathway is activated when IL-6 is released from cells of the immune system in response to inflammatory conditions (Schmidt-Arras and Rose-John, 2016). The second enriched pathway in CH, the acute phase response pathway, is an early step in fighting infection and serves to initiate inflammation upon the detection of pathogens or injury (Ceciliani et al., 2012). In support of the immune-related findings in CH, nine of the ten canonical pathways enriched across all diet-breed comparisons for HF steers were also related to immune function or autoimmunity. Previous work by Salleh et al. (2017) reported similar findings whereby they observed that pathways related to immune function were enriched in RFI divergent dairy cattle. In beef cattle, several studies have reported enrichment of immune-related pathways in RFI-divergent cattle (Zarek et al., 2017, Paradis et al., 2015). These results, coupled with the findings of the current study, highlight the role of the immune system in efficient feed usage.

Of the individual genes identified as differentially expressed in more than one diet, *GADD45G* and *HP* have been identified as associated with immune-related functions. In the present study, *GADD45G* expression was upregulated in low RFI steers offered the H1 diet, while its expression was downregulated in low RFI CH steers fed the ZG diet. The increased expression of *GADD45G* in low-RFI steers offered a high-concentrate diet, which is different from previous observations where *GADD45G* was downregulated when Nellore cattle were offered a forage-based diet (Alexandre et al., 2015). However, it has been suggested that liver inflammation may occur when an animal is fed a high-concentrate diet (Alexandre et al., 2015, Ametaj et al., 2009). This may account for the increased expression of *GADD45G* during the H1 phase. The downregulation of *GADD45G* in low-RFI steers offered the ZG diet may indicate that low-RFI steers experience less inflammation than their high-RFI counterparts when offered a grass diet. Previous work in Canadian cattle identified *HP* as downregulated in the liver of low-RFI Angus steers offered a high-concentrate diet (Mukiibi et al., 2018), however in the present study *HP* expression was increased in low-RFI CH steers offered both the H1 and ZG diets.

Several groups have suggested that increased inflammation leads to poor feed efficiency due to increased energy expended fighting infection, or other pro-inflammatory challenges (Vigors et al., 2016, Kern et al., 2016). However, others have suggested that increased expression of pro-inflammatory genes enables cattle to respond more efficiently to immune challenges and therefore use less energy combating chronic infection (Paradis et al., 2015, Weber et al., 2016). Our results indicate that diet may also play a key role in the effect of the immune system on RFI status by causing a genotype-by-environment interaction, whereby feed type causes inflammation or immune challenge.

3.5.2. Lipid metabolism

The final DEG identified in both H1 and ZG diets for CH was *MID1IP1*, a gene required for fatty acid and lipid synthesis (Kim et al., 2010). *MID1IP1* was observed to be downregulated in low-RFI CH steers offered H1 and ZG diets. Downregulation of *MID1IP1* in low-RFI CH cattle is in agreement with previous work carried out in

Canadian beef cattle, where it was observed that low-RFI steers displayed lower levels of hepatic lipid synthesis than high-RFI steers (Mukiibi et al., 2018). The same authors suggested that decreased lipid synthesis may be due to efficient cattle partitioning greater energy to muscle deposition than fat. Lipid metabolism was also observed to be an enriched biological function in all breed-diet comparisons in this study, illustrating the integral role that this pathway plays in RFI divergence as has been previously reported in other hepatic transcriptome studies (Chen et al., 2011, Alexandre et al., 2015, Tizioto et al., 2015, Mukiibi et al., 2018).

Diacylglycerol acyltransferase (DGAT), a gene within the lipid metabolism biological function, was identified as downregulated in low-RFI CH steers offered the H1 diet. Similarly, Salleh et al. (2017) found this gene to be down-regulated in the hepatic transcriptome of low-RFI Holstein cattle. Contrastingly, *insulin receptor substrate 2 (IRS2)* was observed to be upregulated in the low-RFI CH steers offered the H1 diet. Previous work in pigs also observed the upregulation of *IRS2* in feed efficient animals (Gondret et al., 2017). *IRS2* knockout mice display increased adiposity and total body fat mass (Kocalis et al., 2014). This potentially indicates that downregulation of *IRS2* observed in the high-RFI CH steers offered the H1 diet may lead to increased energy partitioned to fat deposition. *Agouti signalling protein (ASIP)* and *synuclein alpha (SNCA)* were both downregulated in the hepatic transcriptome of CH steers offered the H2 diet. Both of these genes have previously been associated with increased lipid synthesis (Liu et al., 2018b, Corominas et al., 2013). These results further support the hypothesis that feed efficient cattle expend less energy for hepatic lipid synthesis than their inefficient counterparts (Mukiibi et al., 2018). Efficient cattle may partition more energy to muscle gain than lipid synthesis, and are therefore more feed efficient (Mukiibi et al., 2018).

3.5.3. Extracellular matrix

Tenascin C (*TNC*) was observed to be upregulated in low-RFI CH steers offered the ZG diet. This gene has previously been identified as upregulated in the liver transcriptome of low-RFI Angus bulls (Chen et al., 2011). Those authors hypothesized that the upregulation of *TNC* may indicate that the liver of low-RFI

cattle exhibit greater cellular organisation than inefficient cattle. Our results support this hypothesis as we also observed the upregulation of *TNC* in efficient animals and that the biological function cellular assembly and organisation was also enriched in all diet-breed comparisons investigated. Previous work investigating differential gene expression in the rumen epithelium found that *tubulin alpha 4a* (*TUBA4A*) was upregulated in low-RFI crossbred steers (Kong et al., 2016b), further supporting the hypothesis that efficient animals exhibit greater extracellular matrix organisation than their inefficient counterparts. However, we have observed that hepatic *TUBA4A*, was downregulated in the low-RFI steers offered the ZG diet. Consequently, further work is required to elucidate the role of extracellular matrix genes in the liver of RFI-divergent cattle, and the role these genes play in feed efficiency.

3.5.4. Oxidative Phosphorylation

Glutathione S-transferase Mu 1 (*GSTM1)* encodes for a member of the glutathione S-transferase family. Chen *et al.,* (2011) observed that *GSTM1* was downregulated in low-RFI Angus bulls. These same authors hypothesized that feed efficient cattle experience less oxidative stress and consequently the mRNA abundance of genes involved in the metabolism of oxidative stress products is reduced. Similarly in the present study, *GSTM1* was identified as a downregulated gene in low-RFI HF steers offered the H1 diet. Our finding of decreased *GSTM1* abundance is in agreement with the hypothesis suggested by Chen et al. (2011) and others who observed that efficient cattle experience less oxidative stress than their inefficient counterparts. This has also been observed in poultry (Iqbal et al., 2005, Bottje and Carstens, 2009). However, Paradis et al. (2015) and Tizioto et al. (2015) observed that *GSTM1* transcript levels were increased in feed efficient crossbred heifers and Nelore steers, respectively. From their findings, Paradis et al. (2015) suggested that low-RFI cattle respond in a more efficient manner to oxidative stress than their high-RFI counterparts. It is possible that observed variation in *GSTM1* expression across studies may represent a genotype-by-environment interaction whereby certain feed efficient animals experience less oxidative stress, while others may be adapted to deal with this stressor in a more effective manner.

3.6. Conclusion

We investigated differential gene expression using RNA-Seq analysis in the liver of CH and HF steers divergent in RFI across three dietary stages, with the goal of identifying genes and pathways associated with RFI across breed and diet. We identified 3 DEGs shared across two diets for CH steers, while two and ten canonical pathways were shared across all diets for CH and HF steers, respectively. Fourteen biological pathways were shared across all diets and both breeds. The identification of physiological processes such as the immune response and lipid metabolism across diet and breed highlight the integral roles of these pathways in the biological processes underlying RFI. Further work investigating genes within these pathways may enable identification of biomarkers for RFI that may be incorporated into genomic assisted breeding programmes, as well as enhancing our understanding of the underlying biology of variation in the RFI trait.

3.7. Supplementary information

Supplementary Table 3.1. Differentially expressed genes for all diet-breed combinations.

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
H1CH	ENSBTAG00000000436	*TNFAIP3*	0.66	1.98E-04
	ENSBTAG00000010219	*KRBA1*	1.39	6.85E-04
	ENSBTAG00000011437	*SIK1*	1.33	4.35E-03
	ENSBTAG00000012019	*IRS2*	1.23	4.35E-03
	ENSBTAG00000034192	*KIAA2012*	-0.53	0.013
	ENSBTAG00000003822	*CROCC*	0.88	0.013
	ENSBTAG00000012896	*METTL7B*	-0.32	0.013
	ENSBTAG00000007865	*GNPDA1*	-0.36	0.013
	ENSBTAG00000046470	*ONECUT1*	0.47	0.013
	ENSBTAG00000005998	*NOCT*	1.86	0.013
	ENSBTAG00000006775	*SLC12A7*	0.46	0.013
	ENSBTAG00000034393	*FAM149A*	-0.26	0.014
	ENSBTAG00000017763	*NFIL3*	-0.41	0.015
	ENSBTAG00000007749	*TONSL*	0.63	0.016
	ENSBTAG00000001631	*KIFC1*	1.16	0.016
	ENSBTAG00000001049	*ANOS1*	0.70	0.018
	ENSBTAG00000021672	*RGS1*	1.30	0.020
	ENSBTAG00000014402	*GIMAP8*	0.60	0.020
	ENSBTAG00000015124	*ARGLU1*	0.35	0.022
	ENSBTAG00000006732	*NRCAM*	1.03	0.022
	ENSBTAG00000005585	ENSBTAG00000005585	-0.90	0.022
	ENSBTAG00000002816	*SARM1*	0.56	0.022

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000014388	*EIF3I*	-0.32	0.022
	ENSBTAG00000016208	*TGM2*	-1.54	0.024
	ENSBTAG00000005430	*MB21D2*	-0.51	0.029
	ENSBTAG00000007116	*ARRDC3*	-0.76	0.029
	ENSBTAG00000002896	*KPNA6*	-0.28	0.030
	ENSBTAG00000011145	*NDUFA4*	-0.36	0.030
	ENSBTAG00000019645	*TFDP1*	0.35	0.030
	ENSBTAG00000002650	*ZPR1*	0.55	0.030
	ENSBTAG00000031001	*MEGF8*	0.53	0.030
	ENSBTAG00000039588	*LOC511617*	0.86	0.032
	ENSBTAG00000001219	ENSBTAG00000001219	-0.84	0.032
	ENSBTAG00000003798	*AMFR*	-0.27	0.032
	ENSBTAG00000006680	*NKIRAS1*	-0.49	0.032
	ENSBTAG00000003935	*TMBIM1*	-0.26	0.032
	ENSBTAG00000040334	ENSBTAG00000040334	0.59	0.032
	ENSBTAG00000021377	*S100A14*	-0.35	0.035
	ENSBTAG00000003665	*NUP210*	0.48	0.036
	ENSBTAG00000011463	*MID1IP1*	-0.67	0.038
	ENSBTAG00000039731	*RND3*	-0.50	0.042
	ENSBTAG00000008837	*CSNK2B*	-0.28	0.042
	ENSBTAG00000048226	ENSBTAG00000048226	0.85	0.046
	ENSBTAG00000019901	*RAD54L*	0.58	0.046
	ENSBTAG00000006784	*PLA2G4B*	0.49	0.046
	ENSBTAG00000004855	*PRDX6*	-0.40	0.046
	ENSBTAG00000017069	*FAM198B*	0.51	0.046
	ENSBTAG00000017536	ENSBTAG00000017536	0.69	0.046

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000019684	*NUDT3*	-0.21	0.046
	ENSBTAG00000008646	*TRMT112*	-0.30	0.046
	ENSBTAG00000006338	*MYCL*	0.93	0.047
	ENSBTAG00000021437	*GPR174*	0.62	0.047
	ENSBTAG00000018010	*ABCA4*	1.15	0.047
	ENSBTAG00000001154	*DGAT2*	-0.53	0.047
	ENSBTAG00000013004	*ITIH5*	-1.03	0.049
	ENSBTAG00000012201	*CYP3A5*	-0.41	0.049
	ENSBTAG00000031590	*SSH3*	0.36	0.050
	ENSBTAG00000031059	*LRRC61*	1.21	0.051
	ENSBTAG00000010481	*UBXN1*	-0.34	0.051
	ENSBTAG00000001288	*MAOB*	-0.30	0.052
	ENSBTAG00000008499	*TROAP*	1.16	0.053
	ENSBTAG00000013880	*WWC1*	-0.72	0.053
	ENSBTAG00000003408	ENSBTAG00000003408	-0.97	0.053
	ENSBTAG00000012121	*NOXA1*	0.72	0.053
	ENSBTAG00000003109	*ITM2B*	-0.26	0.053
	ENSBTAG00000018825	*SSH1*	0.39	0.053
	ENSBTAG00000006354	*HP*	2.25	0.053
	ENSBTAG00000011237	*PKHD1*	0.65	0.053
	ENSBTAG00000004782	*PTPRCAP*	0.52	0.053
	ENSBTAG00000010349	*IL1A*	1.01	0.055
	ENSBTAG00000018588	*TMBIM6*	-0.33	0.059
	ENSBTAG00000014380	*MCM2*	0.55	0.067
	ENSBTAG00000017852	*ALDH9A1*	-0.26	0.069
	ENSBTAG00000037377	*ABHD14B*	-0.30	0.069

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000016592	*CAPN15*	0.58	0.069
	ENSBTAG00000013556	*UNC13D*	0.47	0.070
	ENSBTAG00000019734	*CD276*	0.47	0.070
	ENSBTAG00000000124	*DHH*	1.07	0.072
	ENSBTAG00000025550	ENSBTAG00000025550	0.71	0.072
	ENSBTAG00000000490	*RTKN*	0.50	0.072
	ENSBTAG00000012178	*NR1D1*	-0.77	0.072
	ENSBTAG00000007362	*XPC*	0.27	0.072
	ENSBTAG00000018101	*AHCY*	-0.33	0.073
	ENSBTAG00000009218	*ANLN*	0.93	0.073
	ENSBTAG00000011110	*ACOT12*	-0.29	0.073
	ENSBTAG00000031252	*CD82*	-0.32	0.073
	ENSBTAG00000010433	*M-SAA3.2*	-2.94	0.075
	ENSBTAG00000010164	*ESPNL*	0.62	0.077
	ENSBTAG00000021162	*CKAP2*	0.96	0.077
	ENSBTAG00000006287	*NEDD9*	0.71	0.077
	ENSBTAG00000031962	*RAB20*	0.43	0.078
	ENSBTAG00000020757	*PCBP2*	-0.20	0.079
	ENSBTAG00000002826	*CLSPN*	0.92	0.084
	ENSBTAG00000010624	*DCTN2*	-0.18	0.084
	ENSBTAG00000039319	*LOC100295883*	-0.32	0.084
	ENSBTAG00000020059	*GEN1*	0.66	0.084
	ENSBTAG00000019805	*RHBDF1*	0.60	0.084
	ENSBTAG00000005572	*ZNF205*	0.30	0.084
	ENSBTAG00000000603	*JAM2*	-0.39	0.086
	ENSBTAG00000019552	*PGRMC1*	-0.32	0.086

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000039374	*IPO5*	-0.26	0.086
	ENSBTAG00000002260	*NCAPD3*	0.23	0.086
	ENSBTAG00000001609	*MAP2K6*	-0.45	0.086
	ENSBTAG00000008192	*PLPPR2*	0.45	0.086
	ENSBTAG00000011423	*SERPINI2*	-4.98	0.088
	ENSBTAG00000013032	*CCNDBP1*	-0.26	0.088
	ENSBTAG00000006609	*ERRFI1*	0.65	0.088
	ENSBTAG00000002451	*TTC39A*	0.92	0.092
	ENSBTAG00000008036	*CELSR1*	1.34	0.092
	ENSBTAG00000003033	*GADD45G*	1.25	0.092
	ENSBTAG00000007737	*UBA52*	-0.34	0.092
	ENSBTAG00000018417	*PSMF1*	-0.32	0.092
	ENSBTAG00000000140	*EPHX1*	-0.38	0.092
	ENSBTAG00000048049	ENSBTAG00000048049	-0.76	0.092
	ENSBTAG00000031778	*HIST1H2BD*	-0.52	0.092
	ENSBTAG00000014831	*PPP1R3C*	-0.64	0.092
	ENSBTAG00000004037	*JUN*	0.37	0.093
	ENSBTAG00000016131	*NCAPG2*	0.46	0.093
	ENSBTAG00000002060	*RPL19*	-0.30	0.094
	ENSBTAG00000014718	*CST6*	0.86	0.094
	ENSBTAG00000026461	*CACNA1H*	0.78	0.094
	ENSBTAG00000010152	*MID1*	-0.37	0.095
	ENSBTAG00000000492	*ASNSD1*	-0.25	0.095
	ENSBTAG00000021446	ENSBTAG00000021446	-0.64	0.095
	ENSBTAG00000002999	*ANAPC15*	-0.38	0.095
	ENSBTAG00000016277	ENSBTAG00000016277	-0.30	0.095

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000003882	*EMC3*	-0.24	0.095
	ENSBTAG00000006882	*IQGAP3*	1.28	0.096
	ENSBTAG00000026232	*TP53INP1*	0.59	0.096
	ENSBTAG00000018451	*PTMS*	-0.28	0.096
	ENSBTAG00000015607	*ERMP1*	0.31	0.096
	ENSBTAG00000008409	*MYC*	0.76	0.096
	ENSBTAG00000033677	*WDR90*	0.57	0.097
	ENSBTAG00000017531	*FETUB*	0.88	0.098
	ENSBTAG00000022155	*FSTL1*	-0.36	0.098
	ENSBTAG00000006370	*CCT3*	-0.28	0.098
	ENSBTAG00000003449	*LOC515624*	0.47	0.098
	ENSBTAG00000025903	*GLDC*	-0.26	0.098
	ENSBTAG00000000413	*NRTN*	1.02	0.098
	ENSBTAG00000021181	*BUB1*	0.99	0.098
	ENSBTAG00000007237	*BUB1B*	1.04	0.098
	ENSBTAG00000005086	*RPRD1B*	-0.19	0.098
	ENSBTAG00000013535	*DFFA*	-0.28	0.098
	ENSBTAG00000011405	*CEP72*	0.54	0.098
	ENSBTAG00000007172	*GOT2*	-0.22	0.098
	ENSBTAG00000001014	*MAPK7*	0.33	0.098
	ENSBTAG00000009984	*SGPL1*	-0.49	0.098
	ENSBTAG00000006543	*EIF3CL*	-0.22	0.098
	ENSBTAG00000002377	*PSMB2*	-0.20	0.098
	ENSBTAG00000005623	*SOAT2*	0.68	0.098
	ENSBTAG00000006883	*EIF4B*	-0.34	0.098
	ENSBTAG00000004258	*EEF2*	-0.28	0.098

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000016683	*NFKBIA*	0.45	0.098
	ENSBTAG00000047319	ENSBTAG00000047319	0.61	0.098
	ENSBTAG00000007187	ENSBTAG00000007187	0.50	0.098
	ENSBTAG00000005664	*YWHAE*	-0.22	0.098
	ENSBTAG00000012761	*PTK7*	0.64	0.098
	ENSBTAG00000021416	*FAM213A*	-0.43	0.098
	ENSBTAG00000018016	*NUPR1*	-0.39	0.098
	ENSBTAG00000026192	ENSBTAG00000026192	0.31	0.099
H2CH	ENSBTAG00000039928	*LOC768255*	-3.55	2.42E-04
	ENSBTAG00000046257	*GIMAP4*	-3.08	6.54E-04
	ENSBTAG00000037613	*UGT2B15*	0.49	0.0225
	ENSBTAG00000034077	*ASIP*	-6.43	0.0302
ZGCH	ENSBTAG00000046026	*SLC39A4*	-2.675	4.58E-09
	ENSBTAG00000038234	*BHMT2*	1.264	7.60E-08
	ENSBTAG00000000575	*TNC*	2.022	2.95E-07
	ENSBTAG00000016032	ENSBTAG00000016032	1.584	2.95E-07
	ENSBTAG00000006921	*ABCA6*	1.155	1.16E-04
	ENSBTAG00000030974	*TUBA4A*	-1.145	9.56E-04
	ENSBTAG00000015208	*CARMIL3*	-4.579	2.81E-03
	ENSBTAG00000009862	*C10H14orf105*	1.916	2.81E-03
	ENSBTAG00000037605	*LOC100848815*	1.117	2.81E-03
	ENSBTAG00000017741	*HACD4*	-2.369	4.09E-03
	ENSBTAG00000039995	*CFH*	1.108	5.30E-03
	ENSBTAG00000010718	*RALGPS2*	1.215	0.011
	ENSBTAG00000035998	*CKB*	-1.505	0.011
	ENSBTAG00000032996	*P4HA1*	1.019	0.011

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000046364	ENSBTAG00000046364	-2.933	0.013
	ENSBTAG00000018073	*TSPO*	-2.131	0.016
	ENSBTAG00000008625	*PLEKHF1*	-1.250	0.016
	ENSBTAG00000044063	*B4GALT6*	0.935	0.016
	ENSBTAG00000022246	*C29H11orf86*	-1.822	0.016
	ENSBTAG00000046677	*RTN4R*	-3.853	0.017
	ENSBTAG00000034435	*NKD2*	-2.522	0.017
	ENSBTAG00000017194	*ASPG*	-2.396	0.017
	ENSBTAG00000004787	*METRNL*	-1.261	0.017
	ENSBTAG00000016864	*LBP*	2.038	0.017
	ENSBTAG00000047766	*G0S2*	-1.001	0.017
	ENSBTAG00000047317	*CL43*	-0.964	0.017
	ENSBTAG00000048034	*GLTPD2*	-1.577	0.020
	ENSBTAG00000016296	*GPD1*	-0.762	0.028
	ENSBTAG00000015209	*MXD4*	-1.688	0.028
	ENSBTAG00000015490	ENSBTAG00000015490	-1.447	0.028
	ENSBTAG00000047896	*COMT*	-1.581	0.028
	ENSBTAG00000003556	*ZFYVE21*	-1.106	0.028
	ENSBTAG00000000815	*EPHA2*	-1.029	0.031
	ENSBTAG00000014848	*NR0B2*	-1.871	0.034
	ENSBTAG00000045910	*TANGO2*	-1.467	0.034
	ENSBTAG00000021842	*FCGR2B*	0.979	0.036
	ENSBTAG00000046450	ENSBTAG00000046450	-1.840	0.036
	ENSBTAG00000008129	*CLSTN3*	-1.805	0.036
	ENSBTAG00000007206	*STRIP2*	1.028	0.036
	ENSBTAG00000004510	*SARDH*	-1.515	0.036

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000023787	*TOR4A*	-4.149	0.036
	ENSBTAG00000020523	*SLC10A5*	1.294	0.036
	ENSBTAG00000018201	*TKFC*	-1.011	0.036
	ENSBTAG00000015113	*CITED4*	-1.591	0.037
	ENSBTAG00000014646	*MZT2B*	-2.669	0.037
	ENSBTAG00000012210	*C5*	0.814	0.037
	ENSBTAG00000018724	*TMEM82*	-1.444	0.037
	ENSBTAG00000006745	*FGG*	0.769	0.037
	ENSBTAG00000021904	ENSBTAG00000021904	-2.865	0.038
	ENSBTAG00000037456	*AHDC1*	-1.805	0.042
	ENSBTAG00000017181	*MACROD1*	-1.037	0.042
	ENSBTAG00000012815	*CLEC12A*	1.081	0.050
	ENSBTAG00000009983	*KIF23*	1.206	0.050
	ENSBTAG00000003470	*TTYH1*	-1.165	0.050
	ENSBTAG00000011881	*TDRD10*	-1.098	0.050
	ENSBTAG00000005272	*DOHH*	-1.343	0.050
	ENSBTAG00000025274	*TUBB4B*	-1.396	0.050
	ENSBTAG00000002110	*DMGDH*	0.894	0.050
	ENSBTAG00000011726	*CRYL1*	-1.157	0.050
	ENSBTAG00000007109	*ASB2*	-1.877	0.050
	ENSBTAG00000007181	*KIFC3*	-1.229	0.050
	ENSBTAG00000007969	*CIDEC*	-1.909	0.050
	ENSBTAG00000014502	*BRAT1*	-1.182	0.050
	ENSBTAG00000046014	*GPR146*	-2.075	0.050
	ENSBTAG00000017722	*F5*	0.684	0.051
	ENSBTAG00000016227	*ASCL1*	-1.655	0.051

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000000292	ENSBTAG00000000292	-0.976	0.052
	ENSBTAG00000011463	*MID1IP1*	-0.939	0.053
	ENSBTAG00000005063	*THEM6*	-2.014	0.053
	ENSBTAG00000009475	*PLXDC2*	0.806	0.053
	ENSBTAG00000004155	*SPATA20*	-1.336	0.053
	ENSBTAG00000011922	*LOC786966*	-1.505	0.053
	ENSBTAG00000006779	*LDHD*	-1.669	0.054
	ENSBTAG00000013578	*CHI3L2*	2.210	0.056
	ENSBTAG00000001473	*ARVCF*	-1.538	0.056
	ENSBTAG00000033669	*IL17RC*	-1.007	0.056
	ENSBTAG00000007375	*MIF*	-2.049	0.056
	ENSBTAG00000031579	*SGO2*	0.990	0.058
	ENSBTAG00000013259	*POLR3A*	1.088	0.058
	ENSBTAG00000046514	*ASMTL*	-1.577	0.058
	ENSBTAG00000008186	*UBXN6*	-1.052	0.059
	ENSBTAG00000017649	*SCML1*	1.548	0.064
	ENSBTAG00000014358	*EVA1B*	-1.845	0.064
	ENSBTAG00000015996	*GPC1*	-1.735	0.064
	ENSBTAG00000005797	*AGXT*	-1.324	0.064
	ENSBTAG00000038241	*REPIN1*	-1.666	0.064
	ENSBTAG00000007001	*SLC26A1*	-1.848	0.064
	ENSBTAG00000001816	*SLC25A42*	-1.044	0.064
	ENSBTAG00000047715	ENSBTAG00000047715	-1.317	0.064
	ENSBTAG00000021008	*ZNF219*	-2.003	0.064
	ENSBTAG00000007834	*PPP1R16A*	-1.994	0.064
	ENSBTAG00000031327	*TRMT61A*	-2.319	0.064

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000010738	*CCL14*	1.808	0.064
	ENSBTAG00000011007	*TTYH2*	-0.687	0.064
	ENSBTAG00000047113	*LOC104975755*	-3.179	0.064
	ENSBTAG00000025441	*HSPA1A*	-1.700	0.064
	ENSBTAG00000038990	*TRAF4*	-1.091	0.064
	ENSBTAG00000000836	*KRT8*	-1.023	0.071
	ENSBTAG00000002224	*UHRF1*	-2.516	0.071
	ENSBTAG00000016254	*HDAC5*	-1.124	0.071
	ENSBTAG00000008551	*TUSC1*	-3.296	0.072
	ENSBTAG00000010772	*TPGS1*	-2.332	0.072
	ENSBTAG00000006515	ENSBTAG00000006515	-1.680	0.072
	ENSBTAG00000012002	*BCAR1*	-1.584	0.072
	ENSBTAG00000017514	*CCND1*	-1.487	0.072
	ENSBTAG00000000962	*STAP1*	0.814	0.072
	ENSBTAG00000003457	*ATF5*	-1.440	0.072
	ENSBTAG00000013792	*UBALD2*	-1.474	0.072
	ENSBTAG00000002467	*FAM173A*	-1.429	0.072
	ENSBTAG00000000647	*SELENOO*	-1.703	0.073
	ENSBTAG00000007043	*SERPINA3*	1.686	0.073
	ENSBTAG00000012189	*TMEM86B*	-1.343	0.075
	ENSBTAG00000006071	*CTTN*	-0.704	0.075
	ENSBTAG00000046339	*VASN*	-1.768	0.077
	ENSBTAG00000001538	ENSBTAG00000001538	-1.680	0.079
	ENSBTAG00000004261	*SPON2*	-1.908	0.079
	ENSBTAG00000020292	*C5H12orf57*	-0.943	0.079
	ENSBTAG00000025809	*ABHD8*	-1.638	0.079

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000040338	*OBSL1*	-1.228	0.080
	ENSBTAG00000038330	*LOC516108*	-1.839	0.080
	ENSBTAG00000009874	*ZXDC*	-1.319	0.080
	ENSBTAG00000005891	*FCRL1*	2.013	0.081
	ENSBTAG00000006354	*HP*	4.876	0.081
	ENSBTAG00000030335	*ALDH4A1*	-1.410	0.083
	ENSBTAG00000007164	*FGFR3*	-1.376	0.083
	ENSBTAG00000011709	*IMPA1*	0.702	0.083
	ENSBTAG00000006938	*PPM1F*	-1.940	0.083
	ENSBTAG00000046875	*AP5B1*	-1.640	0.083
	ENSBTAG00000008497	*RGS14*	-1.021	0.083
	ENSBTAG00000008237	*TOLLIP*	-1.479	0.083
	ENSBTAG00000006439	*DOCK11*	0.771	0.086
	ENSBTAG00000013996	*SH3BP2*	-0.978	0.086
	ENSBTAG00000039643	*LOC785762*	0.608	0.090
	ENSBTAG00000009211	*UBE2S*	-1.246	0.090
	ENSBTAG00000003446	*EPHB4*	-0.893	0.093
	ENSBTAG00000012044	*RPL13*	-0.949	0.094
	ENSBTAG00000008778	*CHST13*	-1.638	0.094
	ENSBTAG00000035858	*MCC*	0.860	0.096
	ENSBTAG00000001638	*FGA*	0.720	0.096
	ENSBTAG00000046987	ENSBTAG00000046987	-1.760	0.096
	ENSBTAG00000033334	*SPATA2L*	-1.737	0.096
	ENSBTAG00000015778	*SASS6*	0.799	0.097
	ENSBTAG00000034349	*IFI44*	0.968	0.097
	ENSBTAG00000004425	*BBS10*	0.782	0.097

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000003033	*GADD45G*	-1.302	0.097
	ENSBTAG00000012398	*APOC3*	-0.727	0.097
	ENSBTAG00000020449	*ETFB*	-0.744	0.097
	ENSBTAG00000012719	*UBTD1*	-1.458	0.097
	ENSBTAG00000046644	*HRAS*	-1.249	0.097
	ENSBTAG00000018284	*KBTBD11*	-3.087	0.097
	ENSBTAG00000007880	*RAB40C*	-1.193	0.097
	ENSBTAG00000011969	*HSPB1*	-1.432	0.097
	ENSBTAG00000012508	*OSGIN1*	-1.443	0.097
	ENSBTAG00000016558	*SPSB3*	-1.341	0.097
	ENSBTAG00000000332	*ERF*	-1.204	0.097
	ENSBTAG00000016552	*NME3*	-1.897	0.099
	ENSBTAG00000011553	*FAHD2A*	-0.770	0.099
	ENSBTAG00000037581	*MZF1*	-1.518	0.099
H1HF	ENSBTAG00000021262	*SNRPD3*	-0.3748	0.0010
	ENSBTAG00000017147	*AK3*	-0.3526	0.0013
	ENSBTAG00000017765	*GSTM1*	-0.8694	0.0061
	ENSBTAG00000000804	*MOB3B*	0.7176	0.0061
	ENSBTAG00000032057	*LOC782233*	-5.4545	0.0061
	ENSBTAG00000014685	*HPRT1*	-0.327	0.011
	ENSBTAG00000032859	ENSBTAG00000032859	-0.659	0.015
	ENSBTAG00000008039	*ACMSD*	-1.037	0.024
	ENSBTAG00000015919	*PARM1*	1.146	0.024
	ENSBTAG00000016881	*ANPEP*	1.095	0.024
	ENSBTAG00000008773	*GUCY2D*	-0.886	0.024
	ENSBTAG00000004288	*GSTA4*	-0.876	0.026

Diet-Breed	Ensembl Gene ID	Gene name	Log2FoldChange	FDR P-value
	ENSBTAG00000018857	*RAB4A*	-0.289	0.026
	ENSBTAG00000007384	*CYTH3*	0.518	0.026
	ENSBTAG00000011720	*HSD17B6*	-0.361	0.027
	ENSBTAG00000009233	*RAC1*	0.349	0.038
	ENSBTAG00000017711	*WHAMM*	0.375	0.058
	ENSBTAG00000002190	*DOCK8*	0.542	0.067
	ENSBTAG00000015387	*RAB40B*	-0.524	0.067
	ENSBTAG00000012887	*FCER1A*	2.198	0.068
	ENSBTAG00000037673	*GSTM1*	-0.873	0.068
	ENSBTAG00000001908	*DLD*	-0.271	0.071
	ENSBTAG00000019956	*CMPK1*	-0.292	0.072
	ENSBTAG00000018026	*LANCL2*	0.416	0.072
	ENSBTAG00000005133	*ANKS6*	0.624	0.099
	ENSBTAG00000031069	*SNX24*	-0.370	0.099
	ENSBTAG00000005133	ENSBTAG00000005133	0.624	0.099
H2HF	ENSBTAG00000024255	*UOX*	0.8517	0.0276
	ENSBTAG00000037743	*C1R*	7.4467	0.0552
	ENSBTAG00000040388	*LOC100295234*	2.9224	0.0552
	ENSBTAG00000024957	*SNCA*	-3.6102	0.0552
	ENSBTAG00000019554	*FBP2*	-3.0631	0.0552
ZGHF	ENSBTAG00000007584	*INPP1*	0.7310	0.0048
	ENSBTAG00000004118	*ALAS1*	-0.9440	0.0741

FDR *P*-value: Benjamini-Hochberg corrected *P*-value.

Supplementary Table 3.2. All canonical pathways identified as significantly enriched by IPA.

Diet-breed combination	Pathway	*P*-value
CHH1	Glycine Betaine Degradation	2.75423E-05
	Acute Phase Response Signalling	7.08E-04
	Hereditary Breast Cancer Signalling	2.14E-03
	EIF2 Signalling	2.69E-03
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	3.55E-03
	Extrinsic Prothrombin Activation Pathway	4.37E-03
	Chronic Myeloid Leukaemia Signalling	4.47E-03
	Germ Cell-Sertoli Cell Junction Signalling	4.68E-03
	Melanoma Signalling	4.90E-03
	GADD45 Signalling	6.17E-03
	Methylglyoxal Degradation VI	6.31E-03
	Ephrin A Signalling	6.31E-03
	PTEN Signalling	6.61E-03
	Endometrial Cancer Signalling	7.59E-03
	Axonal Guidance Signalling	7.59E-03
	IL-6 Signalling	8.51E-03
	ErbB2-ErbB3 Signalling	9.33E-03
	14-3-3-mediated Signalling	9.33E-03
	Xenobiotic Metabolism Signalling	9.77E-03
	GM-CSF Signalling	0.011
	Aryl Hydrocarbon Receptor Signalling	0.012
	Toll-like Receptor Signalling	0.012
	Non-Small Cell Lung Cancer Signalling	0.012
	Asparagine Degradation I	0.013
	Glycine Biosynthesis III	0.013
	LPS/IL-1 Mediated Inhibition of RXR Function	0.013
	FcγRIIB Signalling in B Lymphocytes	0.013
	Estrogen-Dependent Breast Cancer Signalling	0.014
	IL-17 Signalling	0.016
	Small Cell Lung Cancer Signalling	0.016
	LPS-stimulated MAPK Signalling	0.017
	Bladder Cancer Signalling	0.018
	HER-2 Signalling in Breast Cancer	0.018
	IL-4 Signalling	0.018
	Proline Degradation	0.019
	D-glucuronate Degradation I	0.019
	Methionine Salvage II (Mammalian)	0.019
	Hypusine Biosynthesis	0.019
	4-hydroxyproline Degradation I	0.019
	Coagulation System	0.020

Diet-breed combination	Pathway	*P*-value
	Acute Myeloid Leukaemia Signalling	0.020
	Dopamine Degradation	0.022
	Complement System	0.022
	Prostate Cancer Signalling	0.023
	TR/RXR Activation	0.023
	FAK Signalling	0.024
	Ephrin Receptor Signalling	0.024
	Glycerol-3-phosphate Shuttle	0.025
	Arginine Degradation I (Arginase Pathway)	0.025
	Sertoli Cell-Sertoli Cell Junction Signalling	0.026
	Thyroid Cancer Signalling	0.026
	VEGF Signalling	0.027
	Intrinsic Prothrombin Activation Pathway	0.028
	Role of NFAT in Regulation of the Immune Response	0.030
	Creatine-phosphate Biosynthesis	0.031
	Eumelanin Biosynthesis	0.031
	Myo-inositol Biosynthesis	0.031
	Pyrimidine Ribonucleotides Interconversion	0.032
	p53 Signalling	0.032
	Telomerase Signalling	0.032
	Dermatan Sulfate Biosynthesis (Late Stages)	0.033
	Paxillin Signalling	0.034
	Gap Junction Signalling	0.034
	Glioma Signalling	0.035
	Pyrimidine Ribonucleotides De Novo Biosynthesis	0.035
	HGF Signalling	0.035
	ERK/MAPK Signalling	0.037
	Glycerol Degradation I	0.037
	Chondroitin Sulfate Biosynthesis (Late Stages)	0.038
	Breast Cancer Regulation by Stathmin1	0.040
	NGF Signalling	0.040
	Natural Killer Cell Signalling	0.041
	Role of Tissue Factor in Cancer	0.043
	PI3K Signalling in B Lymphocytes	0.048
	Integrin Signalling	0.049
	Chondroitin Sulfate Biosynthesis	0.049
	Regulation of Cellular Mechanics by Calpain Protease	0.049
CHH2	IL-10 Signalling	1.86E-03
	IL-6 Signalling	2.88E-03
	Circadian Rhythm Signalling	3.63E-03
	Sirtuin Signalling Pathway	3.63E-03
	Clathrin-mediated Endocytosis Signalling	5.89E-03
	The Visual Cycle	5.89E-03

Diet-breed combination	Pathway	*P*-value
	Osteoarthritis Pathway	6.92E-03
	PPARα/RXRα Activation	7.08E-03
	FGF Signalling	7.24E-03
	LXR/RXR Activation	7.76E-03
	LPS/IL-1 Mediated Inhibition of RXR Function	9.33E-03
	RANK Signalling in Osteoclasts	0.012
	Neurotrophin/TRK Signalling	0.015
	2-amino-3-carboxymuconate Semialdehyde Degradation to Glutaryl-CoA	0.019
	UDP-N-acetyl-D-galactosamine Biosynthesis I	0.019
	BER pathway	0.020
	Fatty Acid β-oxidation I	0.022
	FLT3 Signalling in Hematopoietic Progenitor Cells	0.024
	Retinoate Biosynthesis I	0.025
	RAR Activation	0.027
	B Cell Receptor Signalling	0.029
	NRF2-mediated Oxidative Stress Response	0.030
	Hepatic Cholestasis	0.032
	FXR/RXR Activation	0.032
	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	0.032
	ILK Signalling	0.032
	Neuroinflammation Signalling Pathway	0.033
	Pyridoxal 5'-phosphate Salvage Pathway	0.034
	Cardiolipin Biosynthesis II	0.037
	Role of IL-17A in Arthritis	0.041
	Acute Phase Response Signalling	0.042
	Retinol Biosynthesis	0.044
	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	0.045
	Role of p14/p19ARF in Tumor Suppression	0.047
	Huntington's Disease Signalling	0.047
	GM-CSF Signalling	0.048
	IGF-1 Signalling	0.049
CHZG	Glycine Betaine Degradation	2.75E-05
	Acute Phase Response Signalling	7.08E-04
	Hereditary Breast Cancer Signalling	2.14E-03
	EIF2 Signalling	2.69E-03
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	3.55E-03
	Extrinsic Prothrombin Activation Pathway	4.37E-03
	Chronic Myeloid Leukemia Signalling	4.47E-03
	Germ Cell-Sertoli Cell Junction Signalling	4.68E-03
	Melanoma Signalling	4.90E-03

Diet-breed combination	Pathway	*P*-value
	GADD45 Signalling	6.17E-03
	Methylglyoxal Degradation VI	6.31E-03
	Ephrin A Signalling	6.31E-03
	PTEN Signalling	6.61E-03
	Endometrial Cancer Signalling	7.59E-03
	Axonal Guidance Signalling	7.59E-03
	IL-6 Signalling	8.51E-03
	ErbB2-ErbB3 Signalling	9.33E-03
	14-3-3-mediated Signalling	9.33E-03
	Xenobiotic Metabolism Signalling	9.77E-03
	GM-CSF Signalling	0.0107
	Aryl Hydrocarbon Receptor Signalling	0.0117
	Toll-like Receptor Signalling	0.0120
	Non-Small Cell Lung Cancer Signalling	0.0123
	Asparagine Degradation I	0.0126
	Glycine Biosynthesis III	0.0126
	LPS/IL-1 Mediated Inhibition of RXR Function	0.0129
	FcγRIIB Signalling in B Lymphocytes	0.0132
	Estrogen-Dependent Breast Cancer Signalling	0.0138
	IL-17 Signalling	0.0162
	Small Cell Lung Cancer Signalling	0.0162
	LPS-stimulated MAPK Signalling	0.0174
	Bladder Cancer Signalling	0.0178
	HER-2 Signalling in Breast Cancer	0.0178
	IL-4 Signalling	0.0182
	Proline Degradation	0.0186
	D-glucoronate Degradation I	0.0186
	Methionine Salvage II (Mammalian)	0.0186
	Hypusine Biosynthesis	0.0186
	4-hydroxyproline Degradation I	0.0186
	Coagulation System	0.0200
	Acute Myeloid Leukaemia Signalling	0.0204
	Dopamine Degradation	0.0224
	Complement System	0.0224
	Prostate Cancer Signalling	0.0229
	TR/RXR Activation	0.0234
	FAK Signalling	0.0240
	Ephrin Receptor Signalling	0.0240
	Glycerol-3-phosphate Shuttle	0.0245
	Arginine Degradation I (Arginase Pathway)	0.0245
	Sertoli Cell-Sertoli Cell Junction Signalling	0.0257
	Thyroid Cancer Signalling	0.0257
	VEGF Signalling	0.0269
	Intrinsic Prothrombin Activation Pathway	0.0282

Diet-breed combination	Pathway	*P*-value
	Role of NFAT in Regulation of the Immune Response	0.0295
	Creatine-phosphate Biosynthesis	0.0309
	Eumelanin Biosynthesis	0.0309
	Myo-inositol Biosynthesis	0.0309
	Pyrimidine Ribonucleotides Interconversion	0.0324
	p53 Signalling	0.0324
	Telomerase Signalling	0.0324
	Dermatan Sulfate Biosynthesis (Late Stages)	0.0331
	Paxillin Signalling	0.0339
	Gap Junction Signalling	0.0339
	Glioma Signalling	0.0347
	Pyrimidine Ribonucleotides De Novo Biosynthesis	0.0347
	HGF Signalling	0.0355
	ERK/MAPK Signalling	0.0372
	Glycerol Degradation I	0.0372
	Chondroitin Sulfate Biosynthesis (Late Stages)	0.0380
	Breast Cancer Regulation by Stathmin1	0.0398
	NGF Signalling	0.0398
	Natural Killer Cell Signalling	0.0407
	Role of Tissue Factor in Cancer	0.0427
	PI3K Signalling in B Lymphocytes	0.0479
	Integrin Signalling	0.0490
	Chondroitin Sulfate Biosynthesis	0.0490
	Regulation of Cellular Mechanics by Calpain Protease	0.0490
HFH1	Glutathione-mediated Detoxification	1.66E-04
	Fatty Acid β-oxidation I	2.04E-04
	Uracil Degradation II (Reductive)	2.34E-04
	Thymine Degradation	2.34E-04
	Stearate Biosynthesis I (Animals)	2.82E-04
	Colorectal Cancer Metastasis Signalling	3.55E-04
	Molecular Mechanisms of Cancer	4.79E-04
	2-oxobutanoate Degradation I	5.75E-04
	Crosstalk between Dendritic Cells and Natural Killer Cells	7.41E-04
	PXR/RXR Activation	9.77E-04
	Fatty Acid Activation	1.29E-03
	RhoGDI Signalling	1.78E-03
	Ceramide Biosynthesis	1.91E-03
	Signaling by Rho Family GTPases	2.45E-03
	Induction of Apoptosis by HIV1	2.63E-03
	Histidine Degradation III	2.95E-03
	iCOS-iCOSL Signalling in T Helper Cells	3.39E-03
	γ-linolenate Biosynthesis II (Animals)	3.80E-03

Diet-breed combination	Pathway	*P*-value
	Mitochondrial L-carnitine Shuttle Pathway	3.80E-03
	Aryl Hydrocarbon Receptor Signalling	3.89E-03
	Tyrosine Biosynthesis IV	4.57E-03
	Valine Degradation I	4.68E-03
	Role of NFAT in Regulation of the Immune Response	7.08E-03
	T Helper Cell Differentiation	7.94E-03
	Methylmalonyl Pathway	8.91E-03
	Phenylalanine Degradation I (Aerobic)	8.91E-03
	Role of Tissue Factor in Cancer	0.01
	Inhibition of Angiogenesis by TSP1	0.0102
	T Cell Receptor Signalling	0.0110
	Autoimmune Thyroid Disease Signalling	0.0110
	IL-8 Signalling	0.0115
	Primary Immunodeficiency Signalling	0.0123
	Choline Biosynthesis III	0.0129
	Sumoylation Pathway	0.0135
	Superpathway of Methionine Degradation	0.0145
	Th1 and Th2 Activation Pathway	0.0151
	IL-17A Signalling in Gastric Cells	0.0158
	Tryptophan Degradation III (Eukaryotic)	0.0158
	Phospholipase C Signalling	0.0158
	Isoleucine Degradation I	0.0162
	γ-glutamyl Cycle	0.0162
	HMGB1 Signalling	0.0162
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.0166
	Adipogenesis pathway	0.0170
	Tec Kinase Signalling	0.0174
	Glycine Cleavage Complex	0.0209
	Glutaryl-CoA Degradation	0.0234
	PKCθ Signaling in T Lymphocytes	0.0240
	Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	0.0257
	mTOR Signalling	0.0282
	Phosphatidylcholine Biosynthesis I	0.0288
	Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	0.0288
	EIF2 Signalling	0.0295
	IL-12 Signalling and Production in Macrophages	0.0302
	CXCR4 Signalling	0.0302
	Type I Diabetes Mellitus Signalling	0.0316
	CCR5 Signalling in Macrophages	0.0339
	Communication between Innate and Adaptive Immune Cells	0.0339

Diet-breed combination	Pathway	*P*-value
	Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	0.0363
	CD28 Signalling in T Helper Cells	0.0363
	GADD45 Signalling	0.0372
	STAT3 Pathway	0.0380
	Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	0.0380
	Glutamate Removal from Folates	0.0398
	2-amino-3-carboxymuconate Semialdehyde Degradation to Glutaryl-CoA	0.0398
	Adenine and Adenosine Salvage VI	0.0398
	Alanine Biosynthesis III	0.0398
	Hematopoiesis from Pluripotent Stem Cells	0.0398
	Th1 Pathway	0.0407
	Renal Cell Carcinoma Signalling	0.0457
	Calcium-induced T Lymphocyte Apoptosis	0.0457
	TNFR1 Signalling	0.0468
	Pathogenesis of Multiple Sclerosis	0.0468
	Cell Cycle: G1/S Checkpoint Regulation	0.0479
HFH2	Th1 and Th2 Activation Pathway	1.514E-07
	Th1 Pathway	2.951E-07
	iCOS-iCOSL Signalling in T Helper Cells	5.495E-07
	Pathogenesis of Multiple Sclerosis	5.888E-07
	Interferon Signalling	8.913E-07
	CD28 Signalling in T Helper Cells	1.288E-06
	Calcium-induced T Lymphocyte Apoptosis	1.66E-06
	ILK Signalling	1.82E-06
	PKCθ Signalling in T Lymphocytes	2.399E-06
	IL-10 Signalling	2.512E-06
	T Helper Cell Differentiation	4.266E-06
	Th2 Pathway	5.888E-06
	Type I Diabetes Mellitus Signalling	6.026E-06
	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	6.457E-06
	Graft-versus-Host Disease Signalling	8.913E-06
	LXR/RXR Activation	1.479E-05
	γ-linolenate Biosynthesis II (Animals)	2.512E-05
	Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	2.818E-05
	CTLA4 Signalling in Cytotoxic T Lymphocytes	6.457E-05
	Role of NFAT in Regulation of the Immune Response	6.607E-05
	B Cell Development	1.20E-04
	Oleate Biosynthesis II (Animals)	1.41E-04
	OX40 Signalling Pathway	1.74E-04

Diet-breed combination	Pathway	*P*-value
	Zymosterol Biosynthesis	2.00E-04
	Leukocyte Extravasation Signalling	2.51E-04
	Nur77 Signalling in T Lymphocytes	3.02E-04
	Phospholipase C Signalling	3.16E-04
	Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	3.31E-04
	Tight Junction Signalling	3.31E-04
	Acute Phase Response Signalling	3.89E-04
	LPS/IL-1 Mediated Inhibition of RXR Function	4.17E-04
	Atherosclerosis Signalling	5.01E-04
	Neuroinflammation Signalling Pathway	5.62E-04
	Autoimmune Thyroid Disease Signalling	6.17E-04
	T Cell Receptor Signalling	6.76E-04
	HMGB1 Signalling	7.24E-04
	Crosstalk between Dendritic Cells and Natural Killer Cells	7.59E-04
	Inflammasome pathway	8.51E-04
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	9.12E-04
	Communication between Innate and Adaptive Immune Cells	1.17E-03
	Sumoylation Pathway	1.26E-03
	Dendritic Cell Maturation	1.26E-03
	TREM1 Signalling	1.29E-03
	Antigen Presentation Pathway	1.38E-03
	Toll-like Receptor Signalling	1.41E-03
	IL-12 Signalling and Production in Macrophages	1.48E-03
	Role of CHK Proteins in Cell Cycle Checkpoint Control	1.51E-03
	RhoA Signalling	1.70E-03
	FXR/RXR Activation	1.86E-03
	Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.91E-03
	Estrogen Biosynthesis	1.95E-03
	NF-κB Signalling	2.29E-03
	Cholesterol Biosynthesis I	2.57E-03
	Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	2.57E-03
	Cholesterol Biosynthesis III (via Desmosterol)	2.57E-03
	Activation of IRF by Cytosolic Pattern Recognition Receptors	2.57E-03
	Allograft Rejection Signalling	2.69E-03
	Stearate Biosynthesis I (Animals)	2.69E-03
	Hepatic Cholestasis	2.88E-03

Diet-breed combination	Pathway	*P*-value
	Superpathway of Cholesterol Biosynthesis	3.09E-03
	Integrin Signalling	3.47E-03
	Dopamine-DARPP32 Feedback in cAMP Signalling	3.47E-03
	Agranulocyte Adhesion and Diapedesis	3.72E-03
	Primary Immunodeficiency Signalling	4.27E-03
	Tetrahydrofolate Salvage from 5,10-methenyltetrahydrofolate	4.57E-03
	Folate Polyglutamylation	4.57E-03
	Wnt/β-catenin Signalling	4.79E-03
	mTOR Signalling	5.01E-03
	Epithelial Adherens Junction Signalling	5.01E-03
	Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	5.13E-03
	Fatty Acid β-oxidation I	5.13E-03
	Sirtuin Signalling Pathway	5.50E-03
	Semaphorin Signalling in Neurons	6.03E-03
	Retinoate Biosynthesis I	6.31E-03
	Sphingosine-1-phosphate Signalling	6.46E-03
	Granulocyte Adhesion and Diapedesis	6.92E-03
	Cholecystokinin/Gastrin-mediated Signalling	6.92E-03
	Type II Diabetes Mellitus Signalling	7.08E-03
	Ethanol Degradation II	8.51E-03
	Ceramide Degradation	9.33E-03
	RAR Activation	9.55E-03
	Oxidative Ethanol Degradation III	0.0105
	Cdc42 Signalling	0.0117
	Xenobiotic Metabolism Signalling	0.0123
	HIPPO Signalling	0.0123
	Histidine Degradation III	0.0123
	Regulation of Actin-based Motility by Rho	0.0145
	Apoptosis Signalling	0.0145
	Germ Cell-Sertoli Cell Junction Signalling	0.0145
	Sphingosine and Sphingosine-1-phosphate Metabolism	0.0155
	Folate Transformations I	0.0155
	Protein Kinase A Signalling	0.0166
	RhoGDI Signalling	0.0166
	Acute Myeloid Leukemia Signalling	0.0166
	Death Receptor Signalling	0.0166
	p38 MAPK Signalling	0.0170
	iNOS Signalling	0.0170
	IL-17A Signalling in Gastric Cells	0.0170
	Ethanol Degradation IV	0.0170
	CCR5 Signalling in Macrophages	0.0182
	PPAR Signalling	0.0182

Diet-breed combination	Pathway	*P*-value
	Glycolysis I	0.0186
	Gluconeogenesis I	0.0186
	Glioma Invasiveness Signalling	0.0191
	Caveolar-mediated Endocytosis Signalling	0.0200
	ATM Signalling	0.0209
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	0.0219
	Colorectal Cancer Metastasis Signalling	0.0219
	Dopamine Receptor Signalling	0.0269
	TNFR2 Signalling	0.0275
	Adrenomedullin Signalling pathway	0.0282
	Molecular Mechanisms of Cancer	0.0282
	CXCR4 Signalling	0.0295
	IL-8 Signalling	0.0302
	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	0.0316
	Antioxidant Action of Vitamin C	0.0316
	Fatty Acid Activation	0.0324
	Bile Acid Biosynthesis, Neutral Pathway	0.0324
	ERK/MAPK Signalling	0.0331
	Actin Nucleation by ARP-WASP Complex	0.0347
	Virus Entry via Endocytic Pathways	0.0347
	Tec Kinase Signalling	0.0347
	Mitochondrial Dysfunction	0.0355
	Cardiac β-adrenergic Signalling	0.0363
	Colanic Acid Building Blocks Biosynthesis	0.0372
	Glucocorticoid Receptor Signalling	0.0380
	Synaptic Long Term Depression	0.0389
	Calcium Signalling	0.0389
	Coagulation System	0.0407
	NF-κB Activation by Viruses	0.0427
	Opioid Signalling Pathway	0.0427
	Sertoli Cell-Sertoli Cell Junction Signalling	0.0437
	UDP-D-xylose and UDP-D-glucuronate Biosynthesis	0.0437
	Palmitate Biosynthesis I (Animals)	0.0437
	Spermidine Biosynthesis I	0.0437
	Fatty Acid Biosynthesis Initiation II	0.0437
	Cardiolipin Biosynthesis II	0.0437
	Formaldehyde Oxidation II (Glutathione-dependent)	0.0437
	Glutamate Biosynthesis II	0.0437
	Glutamate Degradation X	0.0437
	Cell Cycle Regulation by BTG Family Proteins	0.0468
	Fc Epsilon RI Signalling	0.0479

Diet-breed combination	Pathway	*P*-value
HFZG	LXR/RXR Activation	9.77E-08
	Superpathway of Citrulline Metabolism	4.07E-05
	Citrulline Biosynthesis	1.95E-04
	PXR/RXR Activation	2.51E-04
	FXR/RXR Activation	3.31E-04
	Xenobiotic Metabolism Signalling	6.46E-04
	Type I Diabetes Mellitus Signalling	8.13E-04
	LPS/IL-1 Mediated Inhibition of RXR Function	9.55E-04
	IL-17 Signalling	1.07E-03
	Arginine Degradation I (Arginase Pathway)	1.07E-03
	Antigen Presentation Pathway	1.70E-03
	Tetrapyrrole Biosynthesis II	1.78E-03
	Urea Cycle	2.63E-03
	Arginine Degradation VI (Arginase 2 Pathway)	2.63E-03
	Role of MAPK Signalling in the Pathogenesis of Influenza	2.95E-03
	EIF2 Signalling	3.31E-03
	Neuroinflammation Signalling Pathway	3.72E-03
	Autoimmune Thyroid Disease Signalling	4.07E-03
	Graft-versus-Host Disease Signalling	4.07E-03
	VDR/RXR Activation	4.17E-03
	IL-17A Signalling in Gastric Cells	4.57E-03
	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	5.13E-03
	Heme Biosynthesis II	6.17E-03
	IL-4 Signalling	7.24E-03
	Aldosterone Signalling in Epithelial Cells	8.13E-03
	Reelin Signalling in Neurons	8.32E-03
	Nur77 Signalling in T Lymphocytes	8.51E-03
	Ceramide Signalling	8.71E-03
	Oncostatin M Signalling	0.0110
	Calcium-induced T Lymphocyte Apoptosis	0.0123
	B Cell Development	0.0126
	Interferon Signalling	0.0126
	Th1 and Th2 Activation Pathway	0.0135
	Cell Cycle Regulation by BTG Family Proteins	0.0138
	IL-10 Signalling	0.0145
	Notch Signalling	0.0148
	γ-glutamyl Cycle	0.0151
	Glioma Invasiveness Signalling	0.0151
	Th2 Pathway	0.0170
	Dendritic Cell Maturation	0.0170
	T Helper Cell Differentiation	0.0174

Diet-breed combination	Pathway	*P*-value
	Retinol Biosynthesis	0.0191
	MIF Regulation of Innate Immunity	0.0204
	Hepatic Cholestasis	0.0224
	D-myo-inositol (1,4,5)-trisphosphate Degradation	0.0245
	Triacylglycerol Biosynthesis	0.0245
	Choline Degradation I	0.0269
	Glycine Degradation (Creatine Biosynthesis)	0.0269
	Allograft Rejection Signalling	0.0288
	Acute Phase Response Signalling	0.0288
	Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	0.0347
	Triacylglycerol Degradation	0.0355
	Fatty Acid α-oxidation	0.0355
	Adipogenesis pathway	0.0363
	Th1 Pathway	0.0372
	Granulocyte Adhesion and Diapedesis	0.0372
	Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	0.0389
	Glutathione Biosynthesis	0.0398
	L-serine Degradation	0.0398
	PPAR Signalling	0.0407
	Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism	0.0417
	Role of NFAT in Regulation of the Immune Response	0.0417
	Glutamate Receptor Signalling	0.0427
	Tryptophan Degradation III (Eukaryotic)	0.0447

CH: Charolais; HF: Holstein-Friesian; H1: High concentrate, phase 1; H2: High concentrate, phase 2; ZG: Zero-grazed grass; *P*-value: Fisher's Exact test *P*-value

Supplementary Table 3.3. The range of *P*-values for the biological functions that were significantly enriched across all dietary phases for low-RFI CH and HF steers

Biological Function	CHH1 *P*-value Range	CHH2 *P*-value Range	CHZG *P*-value Range	HFH1 *P*-value Range	HFH2 *P*-value Range	HFZG *P*-value Range
Cell cycle	3.87E-07-6.42E-03	1.06E-03-1.18E-02	1.31E-04-4.5E-03	1.31E-04-5.99E-04	1.56E-06-7.08E-05	1.88E-04-1.88E-04
Cell death and survival	4.5E-07-6.42E-03	9.56E-04-1.39E-02	1.63E-04-4.11E-03	2.95E-08-1.22E-03	2.54E-14-7.3E-05	9.73E-08-6.66E-04
Cell morphology	1.15E-04-6.42E-03	2.29E-05-1.38E-02	2.37E-04-5.08E-03	8.26E-07-1.24E-03	2.09E-08-6.12E-05	3.4E-04-5.58E-04
Cell-to-cell signalling and interaction	8.37E-04-6.42E-03	1.79E-04-1.2E-02	2.84E-04-5.08E-03	4.22E-06-1.22E-03	3.74E-10-7.01E-05	1.1E-07-7.18E-04
Cellular compromise	1.76E-04-6.42E-03	1.06E-03-1.41E-02	3.93E-04-2.52E-03	3.06E-04-1.23E-03	1.53E-06-4.14E-05	1.36E-04-2.86E-04
Cellular development	1.25E-04-6.42E-03	3.54E-04-1.18E-02	5.35E-04-5.08E-03	1.3E-08-1.1E-03	2.38E-07-6.47E-05	2.48E-05-5.13E-04
Cellular function and maintenance	4.61E-04-6.42E-03	6.06E-05-1.41E-02	1.15E-03-5.02E-03	1.43E-07-1.06E-03	5.38E-11-6.61E-05	8.43E-10-6.29E-04
Cellular growth and proliferation	1.25E-04-6.42E-03	1E-03-1.42E-02	2.52E-03-4.87E-03	1.3E-08-1.22E-03	1.77E-07-6.47E-05	4.67E-06-6.58E-04
Cellular movement	1.17E-04-6.42E-03	1.36E-05-1.42E-02	1.36E-05-5.08E-03	1.75E-06-1.23E-03	7.11E-11-9.41E-05	3.68E-09-7.18E-04
Lipid metabolism	8.04E-05-6.42E-03	4.53E-06-1.18E-02	9.13E-04-4.5E-03	2.93E-07-1.08E-03	3.36E-11-7.85E-05	2.35E-09-5.58E-04
Molecular transport	8.04E-05-6.42E-03	4.53E-06-1.38E-02	9.13E-04-3.73E-03	4.25E-05-1.11E-03	1.33E-06-8.56E-05	2.35E-09-6.03E-04
Small Molecule Biochemistry	8.04E-05-6.42E-03	4.53E-06-1.38E-02	3.97E-05-4.5E-03	2.21E-08-1.08E-03	3.36E-11-7.85E-05	2.35E-09-7.11E-04
Cellular assembly and organisation	1.15E-04-6.42E-03	1.06E-03-8.6E-03	1.39E-03-2.52E-03	8.26E-07-2.67E-04	2.45E-06-6.47E-05	2.5E-04-4.75E-04
Carbohydrate metabolism	6E-05-6.42E-03	1.38E-04-1.25E-02	1.09E-03-1.09E-03	9.22E-04-9.22E-04	4.78E-07-9.31E-05	1.8E-07-3.68E-04

CH: Charolais; HF: Holstein-Friesian; H1: High-concentrate, phase 1; H2: High concentrate, phase 2; ZG: Zero-grazed grass; *P*-value range: Range of Fisher's Exact Test *P*-values.

Supplementary Table 3.4. All biological functions identified as significantly enriched by IPA for each respective diet-breed combination.

Diet-breed combination	Biological functions
CHH1	Cell Cycle
	Cell Death and Survival
	Gene Expression
	Lipid Metabolism
	Molecular Transport
	Small Molecule Biochemistry
	Carbohydrate Metabolism
	Cellular Movement
	Cellular Development
	Cellular Growth and Proliferation
	Cell Morphology
	Cellular Assembly and Organization
	Cellular Compromise
	DNA Replication, Recombination, and Repair
	Cellular Function and Maintenance
	Protein Synthesis
	Cell-To-Cell Signalling and Interaction
	Cellular Response to Therapeutics
	Drug Metabolism
	Protein Trafficking
	Energy Production
	Nucleic Acid Metabolism
	Cell Signalling
	RNA Damage and Repair
	RNA Post-Transcriptional Modification
	Amino Acid Metabolism
	Free Radical Scavenging
	Vitamin and Mineral Metabolism
CHH2	Lipid Metabolism
	Molecular Transport
	Small Molecule Biochemistry
	Cellular Movement
	Cell Morphology
	Cellular Function and Maintenance
	Carbohydrate Metabolism
	Cell-To-Cell Signalling and Interaction
	Cellular Development
	Energy Production
	Cellular Growth and Proliferation
	Cell Death and Survival
	Cell Cycle
	Cellular Compromise

Diet-breed combination	Biological functions
	Gene Expression
	Cellular Assembly and Organization
	DNA Replication, Recombination, and Repair
	Amino Acid Metabolism
	Drug Metabolism
	Free Radical Scavenging
	RNA Post-Transcriptional Modification
	Cell Signalling
	Vitamin and Mineral Metabolism
	Protein Synthesis
	Nucleic Acid Metabolism
CHZG	Cellular Movement
	Small Molecule Biochemistry
	Amino Acid Metabolism
	Cell Cycle
	Cell Death and Survival
	Cell Morphology
	Cell-To-Cell Signaling and Interaction
	Cellular Compromise
	DNA Replication, Recombination, and Repair
	Lipid Metabolism
	Molecular Transport
	Cellular Development
	Cellular Function and Maintenance
	Carbohydrate Metabolism
	Cellular Assembly and Organization
	Cellular Growth and Proliferation
HFH1	Cellular Development
	Cellular Growth and Proliferation
	Cell Death and Survival
	Amino Acid Metabolism
	Small Molecule Biochemistry
	Lipid Metabolism
	Cellular Function and Maintenance
	Cellular Movement
	Cell Morphology
	Cellular Assembly and Organization
	Cell-To-Cell Signalling and Interaction
	Nucleic Acid Metabolism
	Molecular Transport
	Cell Signalling
	Gene Expression
	Energy Production
	Post-Translational Modification
	Protein Synthesis

Diet-breed combination	Biological functions
	Cell Cycle
	Vitamin and Mineral Metabolism
	Carbohydrate Metabolism
	Drug Metabolism
	Free Radical Scavenging
	Cellular Compromise
	Cellular Response to Therapeutics
	Protein Trafficking
HFH2	Cell Death and Survival
	Cellular Function and Maintenance
	Lipid Metabolism
	Small Molecule Biochemistry
	Cellular Movement
	Cell-To-Cell Signaling and Interaction
	Cell Morphology
	Cellular Growth and Proliferation
	Cellular Development
	Carbohydrate Metabolism
	Free Radical Scavenging
	Molecular Transport
	Cell Cycle
	Gene Expression
	Cellular Compromise
	Energy Production
	Cell Signaling
	Vitamin and Mineral Metabolism
	Protein Synthesis
	Cellular Assembly and Organization
	Antigen Presentation
	Nucleic Acid Metabolism
	Post-Translational Modification
	Protein Trafficking
HFZG	Cellular Function and Maintenance
	Cellular Movement
	Lipid Metabolism
	Molecular Transport
	Small Molecule Biochemistry
	Cell-To-Cell Signaling and Interaction
	Carbohydrate Metabolism
	Cell Death and Survival
	Protein Synthesis
	Cellular Growth and Proliferation
	Amino Acid Metabolism
	Post-Translational Modification
	Cellular Development

Diet-breed combination	Biological functions
	Cell Signalling
	Free Radical Scavenging
	Nucleic Acid Metabolism
	Vitamin and Mineral Metabolism
	Cellular Compromise
	Cell Cycle
	Cellular Assembly and Organization
	Cell Morphology
	Drug Metabolism
	RNA Post-Transcriptional Modification

CH: Charolais; HF: Holstein-Friesian; H1: High concentrate, phase 1; High concentrate, phase 2; ZG: Zero-grazed grass.

Chapter 4

Network biology approaches to identify candidate genes and physiological processes associated with feed efficiency in the hepatic transcriptome of beef steers offered several dietary phases.

Preamble to Chapter 4: Statement of contribution

This chapter reports work investigating gene co-expression in two breeds of beef steer offered three differing dietary phases throughout their lifetime. The animal model employed is as per Chapter 3. The animal model was generated by Prof. David Kenny, Dr. Mark McGee, Dr. Claire Fitzsimons and Séan Coyle, who also collected the biopsies for this investigation. I generated all required gene expression data required for this work. I also carried out bioinformatic analysis reported in this work. This chapter will be prepared as a manuscript for submission to a peer reviewed journal.

4. Network biology approaches to identify candidate genes and physiological processes associated with feed efficiency in the hepatic transcriptome of beef steers offered several dietary phases.

4.1. Introduction

Feed costs account for more than 70% of fixed expenditure in beef production systems, consequently a reduction in feed costs while maintaining expenditure would result in improved profits (Finneran et al., 2010, Kenny et al., 2018). One potential method by which feed costs could be decreased while output is maintained is through the incorporation of feed efficient animals into the herd (Cantalapiedra-Hijar et al., 2018).

Residual feed intake (RFI) is a common measure of feed efficiency, defined as the difference between an animal's actual and predicted feed intake (Koch et al., 1963). Low-RFI (efficient) animals consume less feed than their high-RFI (inefficient) counterparts, therefore the incorporation of low-RFI cattle into herds would improve profits (Moore et al., 2009). RFI is not widely implemented in beef cattle breeding programmes worldwide due to the expense associated with measuring this trait (Nielsen et al., 2013). However, RFI's moderate-to-high estimated heritability, in the range of 0.26-0.54 (Crowley et al., 2011), indicates that this trait may be ideal for genomic selection. This would circumvent the need for expensive and time consuming feeding trials (Cole and VanRaden, 2017).

For genomic selection to be utilised, genetic markers such as single nucleotide polymorphisms (SNPs) associated with RFI must be identified. Once identified these variants may be included on genotyping chips, such as the Irish custom genotyping chip which is known as the International Dairy and Beef (IDB) microarray chip (Mullen et al., 2013). These genetic markers must be robust across breeds and diet offered, as it has been shown that cattle may re-rank in terms of RFI depending on the diet offered (Durunna et al., 2012, Coyle et al., 2016) and that certain breeds are more efficient than others (Crowley et al., 2010). This represents a particular challenge in

beef production systems as cattle are often raised on a grass-based diet, which is followed by a finishing period where cattle consume a high-concentrate diet (Mulliniks et al., 2015). Furthermore in Ireland, there are a multiplicity of breeds, and their crossbreds, present in beef production systems (Wickham et al., 2012). Therefore, in order for genetic variants to be included in the Irish national genomic-assisted breeding programme, they must be robust across the dietary phases, regardless of breed.

One method of identifying genetic markers for RFI is by utilising the candidate gene approach (Tabor et al., 2002). This approach involves identifying genes associated with the trait of interest, which can then be interrogated for SNPs to be included in genomic assisted breeding programmes (Mullen et al., 2012). RNA-Seq studies have been widely used to identify candidate genes for feed efficiency in cattle (Mukiibi et al., 2018, Salleh et al., 2017, Tizioto et al., 2016, Kong et al., 2016b). However, RNA-Seq studies focus only on differentially expressed genes rather than providing insight into the entire biological system (Hudson et al., 2012). Co-expression network analysis integrates gene expression data and groups genes into modules based on co-expression enabling insight into gene-gene interactions (Zhang and Horvath, 2005). This results in clusters of genes which may function together to cause phenotypic variation (Langfelder and Horvath, 2008).

Identification of biological pathways enriched in the gene modules of interest may provide insight into the underlying physiological control of RFI, furthering our understanding of this trait. Additionally, these gene modules can be investigated for the presence of highly inter-connected genes, known as hub genes. It has been suggested that hub genes are important in regulating the expression of other genes within their module, and consequently are candidate biomarkers for further study (Suravajhala et al., 2016, Kogelman et al., 2014). This approach has been used previously in bovine studies to identify genes and pathways exerting control over feed efficiency. For example, Kong et al. (2016b) identified three gene modules significantly associated with RFI in the rumen papillae of crossbred steers. These modules were enriched for processes such as protein synthesis and oxidative

phosphorylation. Carrying out analysis of the hepatic transcriptome of Nellore steers, Alexandre et al. (2015) identified four modules associated with RFI. These same authors identified eight genes within these modules as putative key regulatory genes and as candidates for further interrogation as potential biomarkers for RFI. While Sun et al. (2018) generated 20 modules significantly associated with RFI from the transcriptome of four tissues: liver, rumen epithelium, adipose tissue and skeletal muscle. They observed that 19 genes were found within each of the most significantly associated modules across each of the four tissues (Sun et al., 2018). However, to-date no studies have interrogated gene co-expression of multiple breeds of RFI-divergent cattle offered varying diets throughout their life.

Consequently, the aims of this study were: (i) to further develop our understanding of the biology of RFI by investigating hub genes and pathways within modules associated with RFI and (ii) to identify hub genes and biological pathways for further interrogation as potential biomarkers for RFI. To achieve these aims we carried out weighted gene co-expression network analysis (WGCNA) on previously generated hepatic gene expression data for RFI-divergent Charolais (CH) and Holstein-Friesian (HF) steers offered multiple diets throughout their life.

4.2. Materials and Methods

All procedures involving animals in this study were conducted under an experimental licence (AE19132/P029) issued by 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.

4.2.1. Animal model

The animal model used within this experiment is as described in Chapter 3 Section 3.3.1 and in the published literature (Coyle et al., 2017, Coyle et al., 2016). Briefly, 90 CH and 77 HF steers were offered varying diets at three stages throughout their life. Steers were first offered a high-concentrate diet (H1), then a zero-grazed grass (ZG)

diet during the growing phase. Cattle were returned to a high concentrate diet (H2) for the finishing phase (Figure 3.1). Following each dietary test, liver biopsies were taken via percutaneous punch biopsy as described by McCarthy et al. (2009) from high and low RFI steers under local anaesthetic (5ml Adrenacaine, Norbrook Laboratories (Ireland Ltd.). Biopsy instruments were sterilized, washed with 70% ethanol and treated with RNaseZap (Ambion, Applera Ireland, Dublin, Ireland). Collected samples were washed in sterile DPBS and snap frozen in liquid nitrogen. All biopsies were stored at -80°C prior to further analysis. 50 mg of the collected biopsy were used for total RNA extraction, and samples with adequate RNA quality were then prepared for sequencing as described in Chapter 3.

4.2.2. Generation of RNA-Sequencing data

To identify modules of co-expressed genes, gene expression data is required. Previously generated RNA-Seq data were utilised in order to carry out gene co-expression analysis. The protocol used to generate these data is as described in Chapter 3. Gene expression data are accessible via the Gene Expression Omnibus repository and can be accessed using the accession number GSE111464.

4.2.3. Gene co-expression analysis

Gene co-expression network analysis was performed using the WGCNA R package (Langfelder and Horvath, 2008). In order to generate modules of similarly expressed genes, gene expression data were provided to WGCNA for each breed and dietary phase. All breeds and dietary phases were analysed individually. To identify modules of co-expressed genes, WGCNA first constructs a similarity matrix which measures similarity between genes with similar expression profiles. The similarity matrix is then transformed into an adjacency matrix by raising the matrix to a soft-thresholding power, denoted by β (Zhang and Horvath, 2005). For all analyses carried out in this study a scale free topology fit (R^2) of 0.9 was chosen and the β employed corresponded to this, as per previous studies (Sun et al., 2018, Ramayo-Caldas et al., 2018).

To define modules of co-expressed genes, WGCNA uses a topological overlap-based dissimilarity measure to group genes into modules, which contain genes whose expression is highly correlated across the samples being studied, by average linkage hierarchical clustering (Zhang and Horvath, 2005). Modules with similar eigengenes, which are representative of the module expression profile, are merged (Langfelder and Horvath, 2007). The resulting modules of co-expressed genes were assigned colour names by the software, and the relationships between these modules and RFI were then calculated by Pearson's correlation. A positive Pearson's correlation indicates that the respective module is associated with increased RFI, while a negative Pearson's correlation indicates that the module is associated with decreased RFI. Modules with statistically significant (*P*-value < 0.05) correlations were selected for further analysis.

4.2.4. Identification of hub genes within modules of interest

Hub genes are highly connected intra-modular genes which tend to have high module membership. Module membership (MM), which measures how correlated a gene is with its module's eigengene, can be used to define a hub gene. MM can be both positive and negative, with genes possessing a MM value close to +1 being positively related to the module eigengene, while genes with values approaching -1 are negatively associated with the module eigengene. A MM closer to an absolute value of 1 indicates a gene is highly connected to the other genes within a module (Langfelder and Horvath, 2008). Consequently, genes with a MM value greater than absolute 0.9 were considered to be hub genes (Kommadath et al., 2014, Horvath and Dong, 2008).

Genes within a module are also assigned a gene significance (GS) measure. This measures how related a specific gene is to the trait under investigation, which is RFI in this case. GS can be either positive or negative, therefore a gene with a high positive GS is highly correlated with increased RFI and vice versa.

4.2.5. Pathway analysis and function enrichment

Functional annotation and pathway analysis was undertaken to investigate biological functions and pathways associated with genes from statistically significant modules as designed by WGCNA. To carry out these analyses, the lists of genes within each statistically significant module were uploaded to Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA) and the Database for Annotation, Visualisation and Integrated Discovery (DAVID, version 6.8) (Huang et al., 2007).

IPA core analysis was performed in order to identify pathways associated with variation in RFI across diet and breed on a module-by-module basis. IPA biological functions were considered to be significantly enriched if the *P*-value of the overlap between the input gene list and the genes within a biological function was deemed to be less than 0.05.

DAVID analysis was carried out to assign genes to pathways as per the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000). DAVID determined enrichment of pathways using Fisher's exact test. Pathways were deemed to be significant if they reached a corrected *P*-value of less than 0.05.

4.3. Results

4.3.1. Gene co-expression analysis

Separate gene co-expression analyses were carried out for each of the diet-breed combinations in the study apart from the CH-ZG combination. The six libraries generated from biopsies taken in this phase were too few to enable accurate gene co-expression analysis using WGCNA. The authors of WGCNA suggest including at least ten samples per analysis (Langfelder, 2017).

For the CH-H1 breed-diet combination a total of 16 gene co-expression modules were detected by WGCNA (Figure 4.1). Four of these modules were significantly associated with variation in RFI. The DarkOrange module was positively associated with RFI (r = 0.55, *P* = 0.02), while the DarkGrey (r = -0.59, *P* = 0.01), Blue (r = -0.49, *P* = 0.04) and Cyan (r = -0.61, *P* = 0.008) modules were all negatively associated with RFI.

Nine modules were identified by WGCNA for the CH steers offered the H2 diet, with two of these being associated with RFI (Figure 4.2). The Black module displayed a negative relationship with RFI (r = -0.58, *P* = 0.04) while the Brown module was positively associated with RFI (r = 0.61, *P* = 0.03).

Twenty and four modules were identified for HF-H1 and HF-ZG breed-diet combinations, respectively. None of these reached statistical significance. For the HF-H2 combination, 20 modules were identified by WGCNA, four of which reached statistical significance (Figure 4.3). Two modules, DarkTurquoise (r = 0.85, *P* = 0.002) and SkyBlue (r = 0.76, *P* = 0.01) were were positively associated with RFI. While both Coral1 (r = -0.74, *P* = 0.02) and Black (r = -0.65, *P* = 0.04) were negatively associated with RFI.

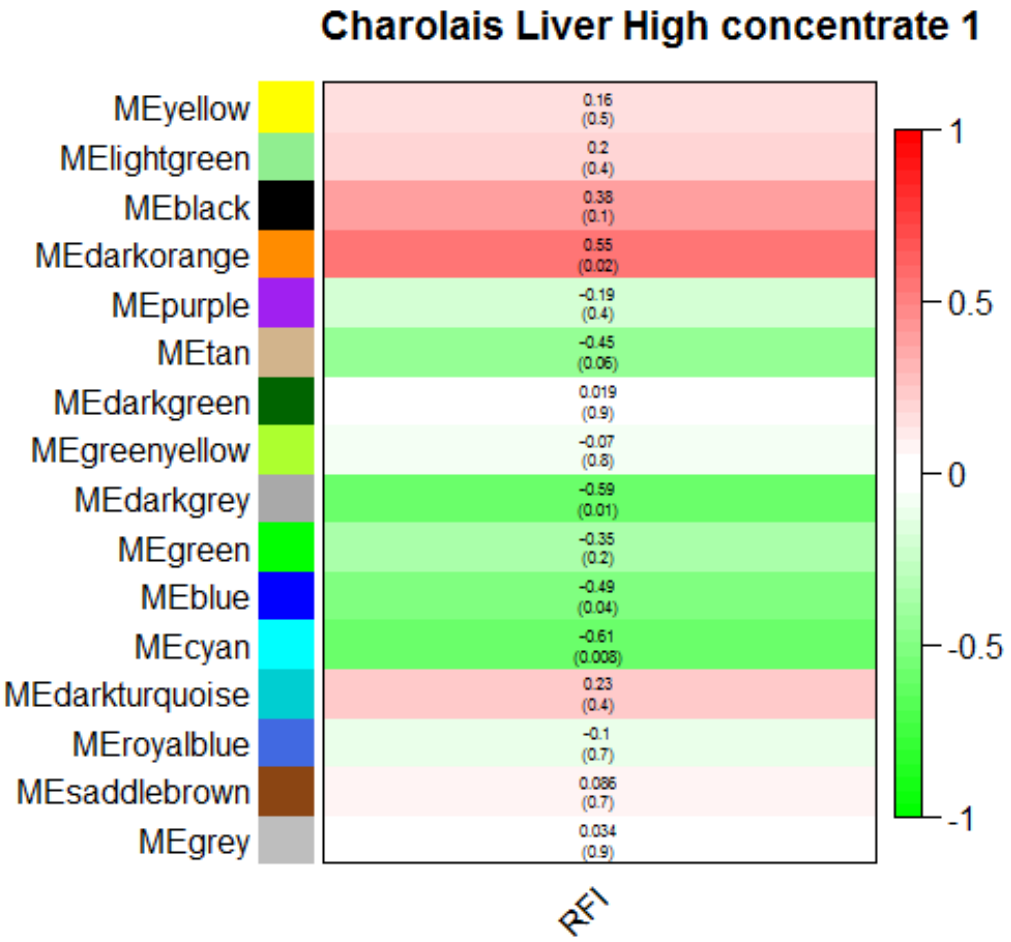

Figure 4.1. Modules of co-expressed genes and correlation coefficients (P-value) of the respective module eigengene and RFI for the Charolais steers offered the high-concentrate 1 diet.

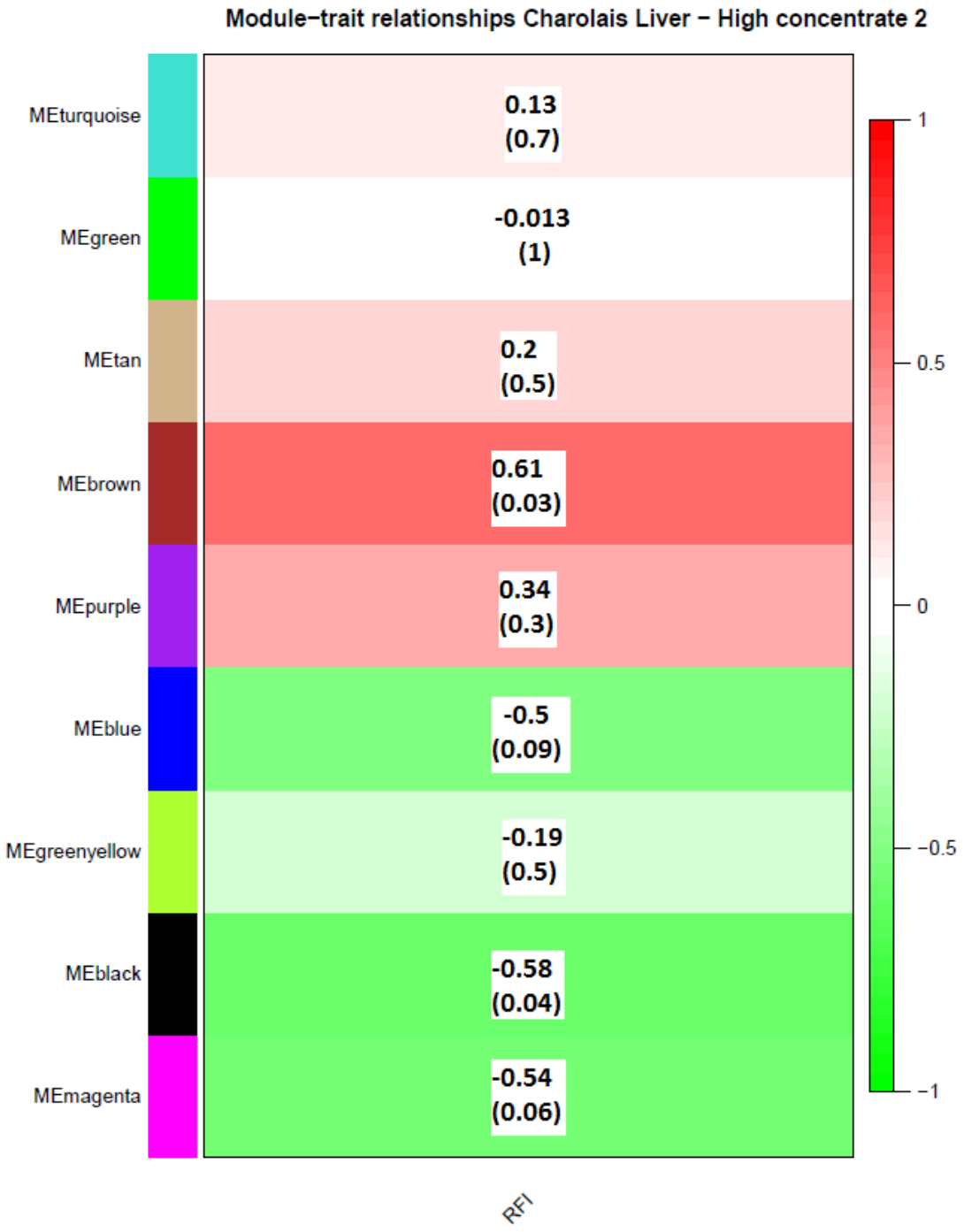

Figure 4.2. Modules of co-expressed genes and correlation coefficients (*P*-value) of the respective module eigengene and RFI for the Charolais steers offered the high-concentrate 2 diet.

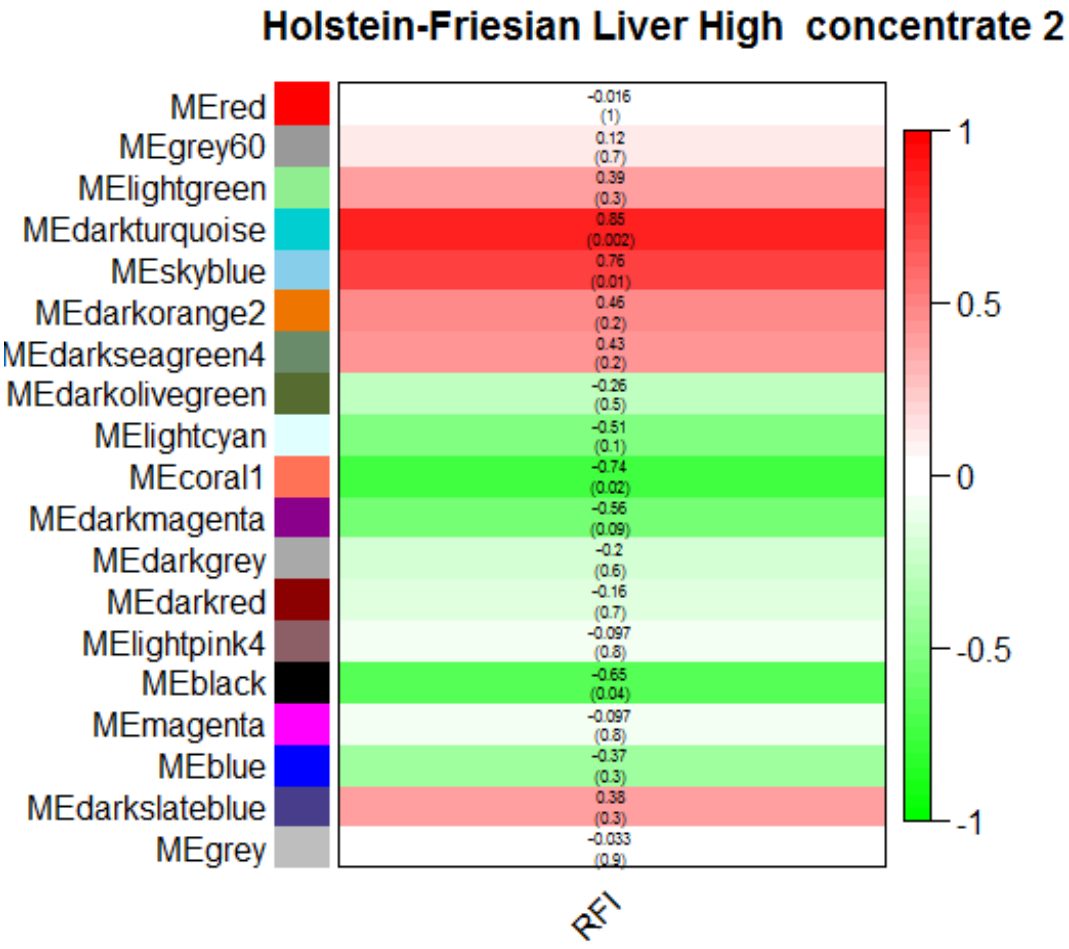

Figure 4.3. Modules of co-expressed genes and correlation coefficients (*P*-value) of the respective module eigengene and RFI for the Holstein-Friesian steers offered the high-concentrate 2 diet.

4.3.2. Identification of hub genes

A total of 71 hub genes were identified within the four statistically significant modules for CH-H1 (Supplementary Table 4.1). Thirty-four hub genes were identified for the statistically significant modules for CH-H2, while 244 hub genes were identified for HF-H2 (Supplementary Table 4.1). No hub gene was expressed in more than one module. The hub genes with a module membership of greater than 0.95 are highlighted in Table 4.1. This cut-off value was chosen in order to enable

tabular visualisation of the top hub genes within a module. Thirty-seven hub genes (from all listed in Supplementary Table 4.1) that have been previously associated with feed efficiency in livestock species are listed in Table 4.2.

Table 4.1. Top hub genes with module membership greater than 0.95 within modules identified as associated with RFI by WGCNA.

Breed-Diet	Module	Ensembl ID	Gene name	MM	GS	Gene function
CH-H1	Cyan	ENSBTAG00000018921	*USP19*	0.97	-0.49	Encodes for an enzyme involved in protein ubiquitination.
		ENSBTAG00000000054	*SNAPC4*	0.96	-0.67	Encodes for an enzyme implicated in transcription.
		ENSBTAG00000017921	*NELFA*	0.95	-0.60	Encodes for a transcription regulating protein.
	Blue	ENSBTAG00000015081	*PAXX*	0.96	-0.41	Encodes for a protein which is implicated in the DNA damage response.
		ENSBTAG00000015097	*CACFD1*	0.96	-0.48	Encodes for a calcium channel protein.
		ENSBTAG00000017750	*RHBDD*	0.96	-0.49	Encodes for a protein which regulates the acute phase response.
		ENSBTAG00000015318	*NECTIN2*	0.96	-0.55	Encodes for a protein which modulates T-cell activity.
		ENSBTAG00000022020	*CLDN5*	0.95	-0.41	Encodes for a protein which is important in creating tight junctions.
CH-H2	Black	ENSBTAG00000006242	*USP9X*	0.95	-0.48	Encodes for a protein which regulates protein degradation and turnover.
		ENSBTAG00000024822	*SETX*	0.95	-0.51	Encodes for an enzyme which regulates RNA polymerase II.
	Brown	ENSBTAG00000011730	*TCAP*	0.95	0.59	Encodes for a protein which functions in assembly of muscle Z-disks.
HF-P4	DarkTurquoise	ENSBTAG00000000011	*TDH*	0.97	0.81	Encodes for an enzyme which mediates amino acid metabolism.
		ENSBTAG00000011217	*DNAJC11*	0.95	0.82	Encodes for a protein required for mitochondrial inner membrane organisation.
	Coral1	ENSBTAG00000001968	*TTC14*	0.97	-0.76	Encodes for a protein with unclear functions, which is involved in nucleic acid binding.
		ENSBTAG00000011889	*NOC4L*	-0.97	0.71	Encodes for a member of a complex which binds RNA.

Breed-Diet	Module	Ensembl ID	Gene name	MM	GS	Gene function
		ENSBTAG00000014807	*DPP8*	0.97	-0.77	Encodes for an enzyme which plays a role in T-cell activation and immune function.
		ENSBTAG00000008167	*TTC3*	0.97	-0.59	Encodes for an ubiquitination protein.
		ENSBTAG00000013651	*DNAJB12*	-0.97	0.73	Encodes for a chaperone protein which aids in degradation of unfolded proteins.
		ENSBTAG00000014616	*SEPSECS*	0.96	-0.77	Encodes for an enzyme which functions in protein synthesis.
		ENSBTAG00000021062	*SMARCD2*	-0.96	0.66	Encodes for a protein which remodels chromatin.
		ENSBTAG00000011727	*ETAA1*	0.96	-0.76	Encodes for a protein which functions to promote the repair of DNA damage sites.
		ENSBTAG00000010059	*C8H9ORF72*	0.96	-0.70	Function unclear, possibly encodes for a protein which is implicated in autophagy.
		ENSBTAG00000045601	*KCTD21*	-0.96	0.66	Encodes for a protein which inhibits cell growth.
		ENSBTAG00000003752	*SLC25A24*	0.95	-0.73	Encodes for a protein which mediates the uptake of adenine nucleotides within the mitochondria.
		ENSBTAG00000031873	*ZSCAN16*	0.95	-0.76	Encodes for a regulator of transcription.
		ENSBTAG00000007825	*MARK2*	-0.95	0.61	Encodes for a serine/threonine kinase which regulates cell polarity and microtubule dynamics.
		ENSBTAG00000017994	*MAX*	-0.95	0.75	Encodes for a regulator of transcription which binds to DNA in a sequence specific manner.
		ENSBTAG00000021225	*DCAF5*	-0.95	0.67	Encodes for a receptor which plays a role in protein ubiquitination.
		ENSBTAG00000032331	*SLC48A1*	-0.95	0.73	Encodes for a protein which regulates intracellular heme availability.
		ENSBTAG00000003419	*EAPP*	0.95	-0.75	Encodes for a protein which regulates cell-cycle progression and may induce apoptosis.
	SkyBlue	ENSBTAG00000003112	*THUMPD3*	-0.98	-0.76	Encodes for an enzyme which binds RNA.

Breed-Diet	Module	Ensembl ID	Gene name	MM	GS	Gene function
		ENSBTAG00000015900	*CNPY3*	0.96	0.79	Encodes for a chaperone protein which is required for Toll-like receptor folding and functions in the immune response.
		ENSBTAG00000017580	*RFX5*	0.95	0.83	Encodes for a transcription activator.
	Black	ENSBTAG00000012644	*UROD*	-0.98	0.63	Encodes for an enzyme which carries out decarboxylation of acetate.
		ENSBTAG00000001792	*C7H19orf70*	-0.97	0.53	Encodes for a protein found in the mitochondrial inner membrane.
		ENSBTAG00000015559	*ERI3*	-0.97	0.72	Encodes for a ribonuclease.
		ENSBTAG00000007809	*PPP1R36*	0.96	-0.57	Encodes for a protein which inhibits phosphatase activity.
		ENSBTAG00000026953	*TRIP11*	0.96	-0.68	Encodes for a transcription factor.
		ENSBTAG00000016230	*BRWD3*	0.95	-0.56	Encodes for a protein which regulates cell shape and cytoskeletal organization.
		ENSBTAG00000016770	*GAPT*	0.95	-0.51	Encodes for a protein which negatively regulates B-cell proliferation.
		ENSBTAG00000014399	*QSER1*	0.95	-0.55	Encodes for a protein with no known function.
		ENSBTAG00000011350	*CCNQ*	-0.95	0.60	Encodes for a protein which activates cyclin-associated kinase CDK10.
		ENSBTAG00000023941	*CPN1*	-0.95	0.48	Encodes for a protein which is implicated in xenobiotic metabolism.
		ENSBTAG00000004874	*AURKAIP1*	-0.95	0.56	Encodes for a protein which downregulates a kinase.
		ENSBTAG00000022777	*CDC42BPA*	0.95	-0.67	Encodes for a kinase which regulates cytoskeletal organisation and cell migration.

MM: Module membership; GS: Gene Significance; CH: Charolais; HF: Holstein-Frieisan; H1: High concentrate diet 1; H2: High concentrate diet 2.

Table 4.2 Hub genes previously associated with RFI or other feed efficiency traits

Breed-Diet	Module	Gene	Gene association with feed efficiency and function
CH-H1	DarkGrey	*DDIT4*	Identified as differentially expressed in the hepatic transcriptome of Kinsella Composite steers by Mukiibi et al. (2018). This gene functions to regulate cell growth.
		NEDD9	Nearby to a SNP associated with feed efficiency in pigs (Onteru et al., 2013). This gene regulates cell adhesion.
	Cyan	*PPP6R2*	Nearby to a SNP associated with RFI in broilers (Liu et al., 2018a). This gene regulates cell cycle progression.
		CRYZ	Identified as within a QTL for DMI by Seabury et al. (2017). This gene may function in xenobiotic metabolism.
	Blue	*TRABD*	Identified as differentially expressed in the rumen papillae of feed efficiency divergent steers (Kern et al., 2016). Implicated in WNT-signalling.
		NME3	Differentially expressed in the rumen papillae of steers divergent for feed efficiency by Kern et al. (2016). Plays a role in synthesis of nucleoside triphosphates.
		CFD	Identified as differentially expressed in the hepatic transcriptome of RFI-divergent Nellore steers (Tizioto et al., 2015). This gene is involved in the complement system which is part of the early immune response.
		ALDH16A1	Identified as nearby to a SNP associated with MBW by Hardie et al. (2017) in dairy cows. This gene detoxifies aldehyde substrates and plays a role in xenobiotic metabolism.
		MRPS2	Differentially expressed in the rumen papillae of RFI-divergent steers (Kern et al., 2016). This gene encodes for a mitochondrial ribosomal protein.
		CTBP1	Identified as nearby to a SNP associated with RFI by Serão et al. (2013b) and as a highly connected gene within a module associated with RFI by

			Alexandre et al. (2015). This gene acts as a transcriptional repressor.
Breed-Diet	**Module**	**Gene**	**Gene association with feed efficiency and function**
		HDAC10	Identified as differentially expressed in the liver of RFI-divergent steers (Tizioto et al., 2015). This gene encodes for a histone deacetylase which plays a role in chromatin modification.
		WRNIP1	Differentially expressed in the rumen papillae for steers divergent in feed efficiency (Kern et al., 2016). This gene encodes for a protein which accumulates at sites of DNA damage and interacts with ubiquitinated proteins.
		MFSD10	Demonstrated to be differentially expressed in the rumen papillae of feed efficiency divergent steers by Kern et al. (2016). This gene encodes for protein which acts as a transporter protein.
		RNH1	Differentially expressed in the rumen papillae of steers divergent for feed efficiency (Kern et al., 2016). This gene produces a protein which functions as an RNase inhibitor.
		FADD	Differentially expressed in the rumen papillae of steers divergent for feed efficiency (Kern et al., 2016). This gene encodes for a protein which mediates apoptosis.
CH-H2	Black	*ANK3*	Differentially expressed in the adipose tissue of pigs divergent in RFI (Louveau et al., 2016). Encodes for a protein which links membrane proteins to the actin cytoskeleton and has a role in functions such as cell motility.
		RNASET2	Differentially expressed in the adipose tissue of RFI-divergent pigs (Gondret et al., 2017). Encodes for an RNase.
	Brown	*LMOD2*	Differentially expressed in the skeletal muscle of broilers divergent for feed efficiency (Kong et al., 2011). Encodes for a gene which regulates actin filament length.
		MYH6	Downregulated in low feed-efficiency broilers (Bottje et al., 2017). Encodes for a myosin isoform.

Breed-Diet	Module	Gene	Gene association with feed efficiency and function
		FLNC	Nearby to a SNP associated with RFI (Hardie et al., 2017). This gene encodes for a filamin protein which crosslinks actin filaments and binds proteins to the cytoskeleton.
		XIRP2	Within a QTL associated with RFI in Angus cattle (Seabury et al., 2017). Encodes for a protein which binds actin and maintains it from depolymerisation.
HF-H2	DarkTurquoise	*TDH*	Differentially expressed in RFI-divergent steers (Chen et al., 2011). Encodes for an enzyme which degrades threonine.
		ATP6V0A1	Identified as within a module associated with RFI following WGCNA analysis of rumen epithelium gene expression data (Kong et al., 2016b). This gene functions to acidify intracellular compartments and functions in oxidative phosphorylation.
		CFL1	Differentially expressed in the rumen epithelium of steers divergent for RFI (Kong et al., 2016b). Encodes for a gene which can polymerize actin and regulates actin dynamics.
	Coral1	*TMEM129*	Nearby to a SNP associated with RFI by Serão et al. (2013b). This gene encodes for a protein implicated in protein degradation.
		PIK3CA	Downregulated in high-RFI dairy cows (Salleh et al., 2017). Participates in cellular signalling often via insulin-receptor substrate proteins.
		HMOX2	Differentially expressed in the rumen papillae of RFI-divergent steers (Kern et al., 2016). Encodes for an oxygenase enzyme which functions in oxidative phosphorylation.
		USP33	Differentially expressed in the adipose tissue of pigs divergent for RFI (Gondret et al., 2017). This gene encodes for a deubiquinating enzyme, and is involved in protein metabolism.
		RASA1	Identified as containing a SNP associated with RFI (Abo-Ismail et al., 2014). Implicated in cellular signalling.

Breed-Diet	Module	Gene	Gene association with feed efficiency and function
		GSTM3	Identified as differentially expressed in the hepatic transcriptome of RFI divergent steers (Chen et al., 2011). This gene encodes for a protein which detoxifies electrophilic compounds.
	SkyBlue	*RFX5*	Differentially expressed in the liver of RFI-divergent dairy cows (Salleh et al., 2017). This gene encodes for a protein which promotes transcription of major histocompatibility complex genes.
	Black	*NSUN2*	Located nearby to a SNP associated with RFI by Serão et al. (2013a). Associated with lipid deposition and RNA translation.
		NIPBL	Within a QTL for RFI identified by Lu et al. (2013b). This gene's protein product has been associated with increased adipogenesis and DNA repair.
		GSTZ1	Identified as differentially expressed in the skeletal muscle of broiler divergent for feed efficiency (Bottje and Kong, 2013). This gene codes for a protein which functions to detoxify electrophilic molecules.
		NDUFB11	Differentially expressed in the liver of feed efficiency divergent pigs (Fu et al., 2017). The protein encoded for by this gene functions in the mitochondrial inner membrane to interact with an electron transfer protein.
		MRPL55	Differentially expressed in the skeletal muscle of broilers divergent for FE (Bottje et al., 2017). This gene encodes for a mitochondrial ribosomal protein which is involved in protein synthesis within the mitochondria.
		COX5B	Differentially expressed in the adipose tissue of pigs divergent in RFI (Fu et al., 2017). This gene encodes for a protein which is part of the mitochondrial respiratory complex.

CH: Charolais; HF: Holstein-Friesian; H1: High concentrate, phase 1; H2: High concentrate, phase 2.

4.3.3. Overlap of DEGs from RNA-Seq and hub genes

A total of ten genes were identified as hub genes in the current study which were also identified as differentially expressed following RNA-Seq analysis carried out in Chapter 3. These genes are listed in Table 4.3.

Table 4.3. Genes designated as hub genes by WGCNA which were also identified as differentially expressed by RNA-Seq analysis carried out in Chapter 3.

Gene	Hub gene within diet-breed module	Gene function
CD82	HF-H2 Coral1	Encodes for a protein which is implicated in cell adhesion.
CSNK2B	CH-H1 DarkOrange	Encodes for a subunit of a protein kinase which functions in a variety of processes including transcription, translation and signal transduction.
FAM173A	CH-H1 Blue	The protein coded for by this gene has no described function.
FGFR3	CH-H1 Blue	Encodes for a growth factor receptor, which once activated leads to cell proliferation.
METTL7B	HF-H2 DarkTurquoise	This gene encodes for a methyltransferase which has been implicated in intracellular transport.
NEDD9	CH-H1 DarkGrey	Encodes for a gene which has functionality in processes including cell adhesion and motility.
NME3	CH-H1 Blue	Encodes for a kinase which is implicated in the synthesis of nucleotide tri-phosphates.
SGO2	HF-H2 DarkTurquoise	Encodes for a gene which is important for centromere stability.
TRMT61A	CH-H1 Blue	Encodes for a methyltransferase which is implicated in gene expression.
ZFYVE21	CH-H1 Blue	Encodes for a protein which functions in cell adhesion and cell motility.

CH: Charolais; HF: Holstein-Friesian; H1: High-concentrate diet 1; H2: High-concentrate diet 2.

4.3.4. Pathway analysis and functional enrichment

For the CH-H1 steers 166, 1685, 322 and 1842 genes were successfully mapped to the IPA database for the DarkGrey, Blue, DarkOrange and Cyan modules, respectively. For the CH-H2 steers 313 genes from the Black module were mapped to the IPA database for analysis, while 989 genes were mapped from the Brown module for analysis. One hundred and fifty-eight, 151, 967 and 985 genes were successfully mapped to the IPA database from the HF-H2 modules DarkTurquoise, SkyBlue, Coral1 and Black, respectively.

Following IPA, enriched canonical pathways for each statistically significant WGCNA module of genes were identified. For the CH steers offered the H1 diet, 37, 52, 51 and 43 canonical pathways were associated with the DarkGrey, Blue, Cyan and DarkOrange modules, respectively. For the CH steers fed the H2 diet 16 and 86 canonical pathways were identified for the Black and Brown modules, respectively. A total of 145, 86, 22 and 5 modules were identified for the Black, Coral1, DarkTurquoise and SkyBlue modules, respectively, for the HF steers offered the H2 diet. The top ten most enriched canonical pathways for each statistically significant module are reported in Table 4.4.

Table 4.4. The top 10 canonical pathways associated with statistically significant WGCNA modules.

Diet-Breed	Module	Pathway	*P*-value
CH-H1	DarkOrange	Complement System	$8.3x10^{-7}$
		Acute Phase Response Signalling	0.0002
		Regulation of eIF4 and p70S6K Signalling	0.0006
		EIF2 Signalling	0.0017
		PI3K/AKT Signalling	0.0029
		Estrogen Receptor Signalling	0.0033
		Assembly of RNA Polymerase II Complex	0.0058
		Hereditary Breast Cancer Signalling	0.0060
		Amyloid Processing	0.0062
		PTEN Signalling	0.0093
	DarkGrey	Bile acid biosynthesis	0.0001
		Salvage Pathways of Pyrimidine Ribonucleotides	0.0008
		PXR/RXR Activation	0.0015
		FXR/RXR Activation	0.0027
		Cell Cycle Regulation by BTG Family Proteins	0.0027
		Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	0.0035
		ERK/MAPK Signalling	0.0046
		Neuregulin Signalling	0.0056
		TR/RXR Activation	0.0064
		ATM Signalling	0.0064
	Blue	Ceramide Signalling	0.0011
		Induction of Apoptosis by HIV1	0.0017
		Mitochondrial Dysfunction	0.0022
		Telomerase Signalling	0.0028
		Protein Ubiquitination Pathway	0.0029
		Telomere Extension by Telomerase	0.0038
		Stearate Biosynthesis I (Animals)	0.0048
		CDK5 Signalling	0.0050
		RAN Signalling	0.0068
		Oxidative Phosphorylation	0.0072
	Cyan	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	$9.12x10^{-6}$
		Superpathway of Inositol Phosphate Compounds	$2.39x10^{-5}$
		Autophagy	$3.09x10^{-5}$
		CD28 Signalling in T Helper Cells	$7.24x10^{-5}$
		Sphingosine-1-phosphate Signalling	$8.13x10^{-5}$
		Clathrin-mediated Endocytosis Signalling	$8.71x10^{-5}$
		mTOR Signalling	$8.71x10^{-5}$
		B-Cell Receptor Signalling	0.0001
		Angiopoietin Signalling	0.0001
		Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	0.0001
CH-H2	Black	TCA Cycle II (Eukaryotic)	0.0044
		Regulation of eIF4 and p70S6K Signalling	0.0081
		Sirtuin Signalling Pathway	0.0083
		Fatty Acid β-oxidation I	0.010
		Autophagy	0.011
		Xanthine and Xanthosine Salvage	0.014

Diet-Breed	Module	Pathway	*P*-value
		Gαs Signalling	0.019
		Virus Entry via Endocytic Pathways	0.023
		UDP-D-xylose and UDP-D-glucuronate Biosynthesis	0.028
		Guanine and Guanosine Salvage I	0.028
	Brown	Calcium Signalling	$1.26x10^{-17}$
		Protein Kinase A Signalling	$1.20x10^{-8}$
		nNOS Signalling in Skeletal Muscle Cells	$1.35x10^{-8}$
		Cardiac β-adrenergic Signalling	$6.46x10^{-8}$
		Cellular Effects of Sildenafil	$2.34x10^{-7}$
		Actin Cytoskeleton Signalling	$5.62x10^{-7}$
		Epithelial Adherens Junction Signalling	$1.12x10^{-5}$
		RhoGDI Signalling	$1.32x10^{-5}$
		Netrin Signalling	$2.51x10^{-5}$
		RhoA Signalling	$2.51x10^{-5}$
HF-H2	Black	Xenobiotic Metabolism Signalling	$3.09x10^{-6}$
		Arginine Biosynthesis IV	$5.25x10^{-5}$
		Glioma Signalling	$7.76x10^{-5}$
		Myc Mediated Apoptosis Signalling	0.0001
		Mouse Embryonic Stem Cell Pluripotency	0.0001
		IGF-1 Signalling	0.0001
		CNTF Signalling	0.0002
		LXR/RXR Activation	0.0003
		NGF Signalling	0.0004
		EIF2 Signalling	0.0005
	Coral1	Mitotic Roles of Polo-Like Kinase	$2.34x10^{-5}$
		Molecular Mechanisms of Cancer	$5.25x10^{-5}$
		ATM Signalling	0.0003
		Breast Cancer Regulation by Stathmin1	0.0004
		ILK Signalling	0.0009
		Sphingosine-1-phosphate Signalling	0.0011
		AMPK Signalling	0.0012
		Relaxin Signalling	0.0014
		Hypoxia Signaling in the Cardiovascular System	0.0014
		Phagosome Formation	0.0017
	DarkTurquoise	GDP-mannose Biosynthesis	0.0007
		Colanic Acid Building Blocks Biosynthesis	0.0042
		Integrin Signalling	0.0045
		Asparagine Biosynthesis I	0.0069
		D-mannose Degradation	0.0069
		Threonine Degradation II	0.0014
		Coenzyme A Biosynthesis	0.0209
		Hypusine Biosynthesis	0.0209
		4-hydroxyproline Degradation I	0.0209
		Glutamate Degradation II	0.0209
	SkyBlue	Formaldehyde Oxidation II (Glutathione-dependent)	0.0138
		Oxidized GTP and dGTP Detoxification	0.0204
		Coagulation System	0.0239
		Pentose Phosphate Pathway (Oxidative Branch)	0.0269
		Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.0407

CH: Charolais; HF: Holstein-Friesian; H1: High concentrate diet 1; H2: High concentrate diet 2; *P*-value: Fisher's exact test *P*-value.

DAVID analysis also revealed that several KEGG pathways were enriched for genes from the statistically significant WGCNA modules. For example, metabolic pathway activation identified as an enriched KEGG pathway in both the CH-H2 Brown module and the HF-H2 Black module. However, no statistically significant KEGG pathways were identified for several modules. The entire list of enriched KEGG terms for each significant module, if applicable, is presented in Table 4.5.

Table 4.5. Significantly enriched KEGG pathways as designated by DAVID analysis.

Diet-Breed	**Module**	**KEGG pathway**	***P*-value**
CH-H1	DarkOrange	Complement and coagulation cascades	0.0015
CH-H2	Brown	Hypertrophic cardiomyopathy	$5.6x10^{-6}$
		Cardiac muscle contraction	$1.7x10^{-5}$
		Dilated cardiomyopathy	$3.3x10^{-5}$
		Oxytocin signalling pathway	$5.7x10^{-4}$
		Adrenergic signalling in cardiomyocytes	$6.3x10^{-4}$
		Arrhythmogenic right ventricular cardiomyopathy	0.0033
		Glucagon signalling pathway	0.0048
		Tight junction	0.022
		Metabolic pathways	0.031
		Viral myocarditis	0.035
		Insulin resistance	0.48
HF-H2	Black	Metabolic pathways	0.0011
		Alzheimer's disease	0.043
	Coral1	Oocyte meiosis	$5.8x10^{-4}$
		AMPK signalling pathway	0.039
		Progesterone mediated oocyte maturation	0.046

CH: Charolais; HF: Holstein-Friesian; H1; High-concentrate diet 1; H2: High-concentrate diet 2; *P*-value: Benjamini-Hochberg corrected *P*-value.

4.4. Discussion

The identification of potential candidate genes and pathways would enable further studies to identify biomarkers of RFI. These could be incorporated into genomic assisted breeding programmes worldwide in order to rapidly and inexpensively

identify feed efficient cattle. However, biomarkers for RFI which are to be incorporated into genomic assisted breeding programmes must be robust across breed and dietary phases which beef cattle may be offered throughout the production cycle. This is of particular importance to the Irish beef production industry as there is a multiplicity of breeds represented, which are offered various feed types throughout their lifetime (Wickham et al., 2012, Coyle et al., 2017, Coyle et al., 2016).

To identify putative candidate genes and pathways of interest that are robust across breed and diet offered, we carried out WGCNA on the liver transcriptome of RFI-divergent CH and HF beef steers fed different diets throughout their life. We identified modules of genes which were significantly associated with RFI across two dietary phases for the CH steers and for one dietary phase for the HF steers.

4.4.1. Mitochondrial efficiency and oxidative phosphorylation

Several hub genes identified in the current study have been associated with feed efficiency and mitochondrial efficiency or oxidative phosphorylation previously. *GSTM3* has previously been identified as differentially expressed in RFI-divergent beef cattle (Chen et al., 2011), and was identified as a hub gene for the HF-H2 Coral1 module. Results reported in Chapter 3 also identified a family member of this gene, *GSTM1*, as differentially expressed in HF steers offered the H1 diet. The identification of this hub gene coupled with the differential expression of a family member in our previous work highlights the importance of this gene family in the response to oxidative stress and its role in RFI-divergence. *ATP6V0A1* and *HMOX2* were identified as differentially expressed in the rumen epithelium of feed efficiency divergent steers (Kong et al., 2016b, Kern et al., 2016), and were identified as hub genes for the HF-H2 DarkTurquoise and Coral1 modules in the current study, respectively. *NDUFB11* and *COX5B* have previously been associated with feed efficiency in pigs (Fu et al., 2017) and *MRPL55* has been associated with divergent feed efficiency in broilers (Bottje et al., 2017), and are hub genes within the HF-H2 Black module.

Functional enrichment analysis of the CH-H1 Blue module revealed that genes within this module were associated with mitochondrial dysfunction and oxidative phosphorylation, while genes within the CH-H2 Black module were enriched for the TCA cycle, which is a biological process implicated in energy production and occurs within mitochondria (Martínez-Reyes et al., 2016). IPA and KEGG analysis both illustrated that genes within the HF-H2 Coral1 module were enriched for the AMPK signalling pathway, which is a pathway activated by the production of reactive oxygen species in the mitochondria (Rabinovitch et al., 2017).

Previous work by our group has illustrated that mitochondrial efficiency may cause variation in RFI (Kelly et al., 2011), while the amount of mitochondria between RFI-divergent cattle does not differ significantly (Kenny et al., 2018). Similarly, Kong et al. (2016b) illustrated that low-RFI steers display increased expression of mitochondrial genes in the rumen epithelium and differences in mitochondrial function has been observed in feed efficiency divergent broilers (Kong et al., 2016a).

Our results, which suggest that genes within modules associated with variation in RFI are enriched for mitochondrial energy production and oxidative phosphorylation across multiple breeds and dietary phases, add further support to the hypothesis that mitochondrial function effects RFI status. Furthermore, due to the enrichment of multiple modules for energy efficiency related genes in modules across three dietary phases, it is possible that genes within these pathways may have potential as candidate genes for RFI across breed and diet. Further interrogation of the pathways and hub genes identified here that have mitochondrial functions is required to both confirm their association with RFI and to identify causal variants for RFI-divergence which may be included in genomic assisted breeding programmes.

4.4.2. The immune response

Several hub genes identified within this study which have been previously associated with feed efficiency in livestock species are part of the immune system. *CFD* has been identified as differentially expressed in the liver of RFI-divergent Nellore steers

(Tizioto et al., 2015) and has been identified as a hub gene within the CH-H1 Blue module. This gene plays an important role in the complement system which is an early response to immune challenge (Stelzer et al., 2016, Merle et al., 2015). While, *RFX5*, a hub gene within the HF-H2 SkyBlue module, was observed to be differentially expressed in the hepatic transcriptome of dairy cows, it encodes a protein required for transcription of genes with immune function (Salleh et al., 2017, Garvie and Boss, 2008). Similarly, several modules were found to be enriched for immune related biological functions following pathway analysis. For example, the CH-H1 DarkOrange module was enriched for the complement system and acute phase response signalling IPA canonical pathways and the complement and coagulation cascade KEGG pathways, all of which are implicated in immune related processes (Stelzer et al., 2016). Other modules were also observed to be enriched for immune related functions such as CDK5 Signalling, which was enriched for within the CH-H1 Blue module; CD28 Signalling in T-helper cells, enriched in the CH-H1 Cyan module and ILK signalling, enriched in the HF-H2 Coral1 module. The enrichment of immune related modules and the identification of hub genes in multiple breeds suggests that this physiological response may be an important mechanism underlying RFI-divergence.

Previous studies have suggested that variation in expression of immune response genes may lead to RFI-divergence. For example, it has been postulated that increased immune response causes increased energy expenditure, and consequently leads to poorer feed efficiency (Kern et al., 2016, Alexandre et al., 2015). However, others have suggested that increased expression of immune related genes enables cattle to more rapidly and energetically-efficiently combat infection and consequently less energy is spent trying to raise an immune response (Paradis et al., 2015, Weber et al., 2016). The results from the current study are supported by our findings in Chapter 3, whereby differentially expressed genes related to immune function were identified as enriched across all dietary phases for both the HF and CH steers by IPA.

Our results, which highlight the immune response as enriched across breeds further supports the hypothesis that the immune system is an important physiological

process in RFI. Further work is required to determine how the immune system specifically effects feed efficiency.

4.4.3. Protein turnover and degradation

Genes and pathways implicated in protein turnover were also present in multiple RFI-associated modules identified in the current study. *WRNIP1* which was identified as a hub gene in the CH-H1 Blue module was previously found to be differentially expressed in the rumen epithelium of feed efficiency-divergent steers (Kern et al., 2016). This gene is implicated in protein ubiquitination (Bish et al., 2008). Additionally, genes within this same module were enriched for the protein ubiquitination IPA biological function. While both *TMEM129* and *USP33* in the HF-H2 Coral1 module play roles in protein turnover and have been associated with RFI previously (Gondret et al., 2017, Serão et al., 2013b).

Protein turnover and degradation are energetically costly processes, which may account for 23% of total energy expenditure (Cantalapiedra-Hijar et al., 2018). Consequently, variation in protein turnover has been suggested as a mechanism resulting in RFI-divergence. For example, Ramayo-Caldas et al. (2018) found that *RNF181* which was differentially expressed in both liver and duodenal tissue of pigs selected for divergent-RFI, plays a role in protein degradation. In cattle, several studies have highlighted genes with roles in protein turnover being differentially expressed between RFI-divergent steers (Chen et al., 2011, Kong et al., 2016b). These results, coupled with the results from the current study indicate that protein turnover is implicated in RFI divergence. The observation from the current study that hub genes with roles in protein turnover have been associated with RFI variation across breed indicates that this physiological function warrants further study for causal variants, which may have utility as biomarkers for RFI across breeds.

4.4.4. Hub genes and pathways associated with muscle contraction

Within the CH-H2 Brown module, which displayed a positive correlation with RFI, four hub genes were identified, all of which had previously been associated with feed

efficiency in livestock species and had biological functions related to muscle contraction. *LMOD2* and *MYH6* have been identified as differentially expressed in broilers divergent for feed efficiency while both *FLNC* and *XIRP2* have been identified as candidate genes associated with RFI divergence in cattle (Kong et al., 2011, Bottje et al., 2017, Hardie et al., 2017, Seabury et al., 2017). Pathway and gene ontology analysis also indicated that the CH-H2 brown module was enriched for genes functioning in processes including actin cytoskeleton signalling, cardiac function and muscle signalling.

Previous work in Charolais steers differing in RFI noted that heart size is correlated with RFI status, whereby more efficient cattle had lighter hearts. These authors suggested that due to the high metabolic demands of cardiac tissue, a lighter heart would consume less energy and therefore the cattle would be able to partition more energy to growth rather than their metabolic needs (Meale et al., 2017). Increased movement, which requires muscle contraction, has previously been associated with increased RFI (Herd and Arthur, 2009). Similarly, increased heart rate has been associated with increased RFI in cows (Hafla et al., 2013). My results which highlight the presence of genes related to muscle contraction and the enrichment of physiological processes related to cardiac function in a module associated with increased RFI, support these previous findings.

4.4.5. Hub genes differentially expressed in RNA-Seq analysis

Of the ten hub genes identified as differentially expressed in the RNA-Seq analysis reported in Chapter 3, only *NEDD9* had previous associations with FE. This gene was identified as nearby to a SNP associated with RFI in pigs (Onteru et al., 2013). *NEDD9* has functions in cell adhesion (Stelzer et al., 2016). Cell adhesion is the process by which cells adhere to each other and to other molecules within the tissue (Alberts, 2015). Variation in cell adhesion has been suggested to cause differences in nutrient absorption, or release, across tissue membranes (Kong et al., 2016b). Genes nearby to SNPs associated with RFI, such as *CD82* and *ZFYVE21*, have previously been found to be involved in cell adhesion (Barendse et al., 2007, Abo-Ismail et al., 2014). Similarly, the cell adhesion biological pathway was enriched for gene

differentially expressed in the rumen epithelium of RFI divergent steers (Kong et al., 2016b). The identification of hub genes identified associated with cell adhesion may also indicate that this process in the liver is an important physiological function with regards to RFI-variation.

4.5. Conclusion

I interrogated hepatic transcriptome data from RFI-divergent CH and HF steers using systems biology approaches in order to identify key genes and pathways associated with RFI divergence. Ten modules were identified across three different breed-diet combinations. Within these modules, 37 hub genes had previously been associated with divergent feed efficiency while ten were observed as differentially expressed in the RNA-Seq analysis carried out in Chapter 3. These hub genes previously associated with RFI and other FE traits may be ideal candidates for biomarker SNP discovery due to their potential role as key regulatory genes. Of particular interest may be *COX5B* and *RFX5* due to the integral role of these genes in their biological pathways and their previous associations with FE. The identification of physiological processes such as protein turnover and mitochondrial function within RFI-associated modules highlights the importance of these pathways in energetic efficiency. Further interrogation of the identified hub genes and the pathways associated with RFI variation may allow identification of genetic variants of RFI which may be included in genomic assisted breeding programmes and may enable further understanding of the processes underlying RFI-variation.

4.6. Supplementary information

Supplementary Table 4.1. Hub genes associated within modules identified as associated with RFI by WGCNA.

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
CH-H1	DarkOrange	ENSBTAG00000008837	*CSNK2B*	0.91	0.69
	DarkGrey	ENSBTAG00000000163	*DDIT4*	0.93	-0.56
		ENSBTAG00000006287	*NEDD9*	0.92	-0.62
	Cyan	ENSBTAG00000018921	*USP19*	0.97	-0.49
		ENSBTAG00000000054	*SNAPC4*	0.96	-0.67
		ENSBTAG00000017921	*NELFA*	0.95	-0.60
		ENSBTAG00000018382	*SMS*	-0.94	0.58
		ENSBTAG00000017074	*ANO8*	0.94	-0.64
		ENSBTAG00000011689	*LENG8*	0.93	-0.65
		ENSBTAG00000018660	*PPP6R2*	0.92	-0.55
		ENSBTAG00000012749	*CUL7*	0.92	-0.59
		ENSBTAG00000020619	*PKD1*	0.92	-0.64
		ENSBTAG00000021157	*PPP6R1*	0.92	-0.69
		ENSBTAG00000008355	*CPSF1*	0.92	-0.68
		ENSBTAG00000020169	*CEPT1*	-0.92	0.48
		ENSBTAG00000017847	*PPP1R12C*	0.92	-0.57
		ENSBTAG00000009761	*ACTR2*	-0.92	0.50
		ENSBTAG00000002774	*PTPN23*	0.92	-0.59
		ENSBTAG00000011064	*ADCK5*	0.91	-0.48
		ENSBTAG00000025320	*AKAP1*	0.91	-0.54
		ENSBTAG00000003162	*CRYZ*	-0.91	0.43
		ENSBTAG00000017289	*MCF2L*	0.91	-0.63
		ENSBTAG00000020617	*TSC2*	0.91	-0.50
		ENSBTAG00000047061	*NDOR1*	0.91	-0.63
		ENSBTAG00000012957	*SERBP1*	-0.91	0.44
		ENSBTAG00000021165	*VPS9D1*	0.91	-0.46
		ENSBTAG00000006124	*MAU2*	0.90	-0.60
		ENSBTAG00000002493	*VPS35*	-0.90	0.45
		ENSBTAG00000012148	*EHMT1*	0.90	-0.52

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
	Blue	ENSBTAG00000015081	*PAXX*	0.96	-0.41
		ENSBTAG00000015097	*CACFD1*	0.96	-0.48
		ENSBTAG00000017750	*RHBDD*	0.96	-0.49
		ENSBTAG00000015318	*NECTIN2*	0.96	-0.55
		ENSBTAG00000022020	*CLDN5*	0.95	-0.41
		ENSBTAG00000047793	*TSSC4*	0.94	-0.48
		ENSBTAG00000014642	*NAPRT*	0.94	-0.54
		ENSBTAG00000001931	*TRABD*	0.94	-0.50
		ENSBTAG00000016810	*PYCR3*	0.93	-0.22
		ENSBTAG00000016552	*NME3*	0.93	-0.49
		ENSBTAG00000039153	*RPS6KA4*	0.92	-0.45
		ENSBTAG00000003556	*ZFYVE21*	0.92	-0.61
		ENSBTAG00000031327	*TRMT61A*	0.92	-0.44
		ENSBTAG00000016553	*EME2*	0.92	-0.63
		ENSBTAG00000002500	*FBXL15*	0.92	-0.44
		ENSBTAG00000007164	*FGFR3*	0.92	-0.72
		ENSBTAG00000048122	*CFD*	0.92	-0.54
		ENSBTAG00000000429	*DGCR2*	0.92	-0.59
		ENSBTAG00000008711	*BOK*	0.92	-0.47
		ENSBTAG00000022027	*DDT*	0.92	-0.39
		ENSBTAG00000038330	Novel cow gene	0.92	-0.32
		ENSBTAG00000002467	*FAM173A*	0.91	-0.36
		ENSBTAG00000048140	*DNAAF5*	0.91	-0.68
		ENSBTAG00000021861	*NELFB*	0.91	-0.36
		ENSBTAG00000019436	*SLC39A13*	0.91	-0.38
		ENSBTAG00000000330	*ALDH16A1*	0.91	-0.61
		ENSBTAG00000005688	*MRPS2*	0.91	-0.57
		ENSBTAG00000000480	*IFRD2*	0.91	-0.45
		ENSBTAG00000008480	*CTBP1*	0.91	-0.58
		ENSBTAG00000013253	*THAP4*	0.91	-0.51
		ENSBTAG00000017925	*LMF2*	0.91	-0.52
		ENSBTAG00000016594	*PIGQ*	0.91	-0.48
		ENSBTAG00000011000	*HDAC10*	0.91	-0.60
		ENSBTAG00000038047	*H2AFX*	0.90	-0.44

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000006166	*WRNIP1*	0.90	-0.68
		ENSBTAG00000012144	*ZMYND19*	0.90	-0.51
		ENSBTAG00000043581	*TXNRD2*	0.90	-0.54
		ENSBTAG00000021130	*MFSD10*	0.90	-0.66
		ENSBTAG00000046869	*RNH1*	0.90	-0.62
		ENSBTAG00000000073	*LRPAP1*	0.90	-0.67
		ENSBTAG00000018274	*FADD*	0.90	-0.33
		ENSBTAG00000006195	*C29H11ORF68*	0.90	-0.29
CH-H2	Black	ENSBTAG00000006242	*USP9X*	0.95	-0.48
		ENSBTAG00000024822	*SETX*	0.95	-0.51
		ENSBTAG00000019052	*ANK3*	0.94	-0.57
		ENSBTAG00000017907	*COG3*	0.93	-0.46
		ENSBTAG00000013013	*GMPS*	0.93	-0.48
		ENSBTAG00000005987	*WDR33*	0.93	-0.34
		ENSBTAG00000004066	*PARP8*	0.92	-0.64
		ENSBTAG00000020782	*CNOT1*	0.92	-0.47
		ENSBTAG00000047036	Novel Cow gene	-0.91	0.36
		ENSBTAG00000014494	*RNASET2*	-0.91	0.39
		ENSBTAG00000012094	*SCAMP3*	-0.91	0.43
	Brown	ENSBTAG00000011730	*TCAP*	0.95	0.59
		ENSBTAG00000042368	*RF00151*	-0.94	-0.54
		ENSBTAG00000010463	Novel Cow gene	0.94	0.58
		ENSBTAG00000005534	*ENO3*	0.93	0.62
		ENSBTAG00000009696	*ACTN2*	0.93	0.68
		ENSBTAG00000002157	*LMOD2*	0.93	0.46
		ENSBTAG00000017509	*MYPN*	0.92	0.57
		ENSBTAG00000009387	*MYOM2*	0.92	0.53
		ENSBTAG00000025136	*MYOZ3*	0.92	0.51
		ENSBTAG00000007247	*NUF2*	0.92	0.43
		ENSBTAG00000046177	*IGFN1*	0.92	0.55
		ENSBTAG00000005353	*DES*	0.92	0.64
		ENSBTAG00000040053	*MYH6*	0.92	0.64
		ENSBTAG00000015470	*SYPL2*	0.92	0.53
		ENSBTAG00000006253	*FLNC*	0.91	0.58

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000015786	*LRRC39*	0.91	0.44
		ENSBTAG00000024929	*PPP1R27*	0.91	0.57
		ENSBTAG00000047155	*C28H10orf71*	0.91	0.72
		ENSBTAG00000021120	*SMYD1*	0.91	0.45
		ENSBTAG00000017743	*XIRP2*	0.91	0.48
		ENSBTAG00000042875	*RF00091*	-0.90	-0.51
		ENSBTAG00000002821	*CILP*	0.90	0.47
		ENSBTAG00000005333	*MB*	0.90	0.60
HF-P4	DarkTurquoise	ENSBTAG00000000011	*TDH*	0.97	0.81
		ENSBTAG00000011217	*DNAJC11*	0.95	0.82
		ENSBTAG00000006920	*ABCF3*	0.94	0.80
		ENSBTAG00000019218	*ATP6V0A1*	0.93	0.77
		ENSBTAG00000021455	*CFL1*	0.93	0.81
		ENSBTAG00000032026	*GMPPB*	0.93	0.84
		ENSBTAG00000031579	*SGO2*	-0.93	-0.93
		ENSBTAG00000011284	*SLC39A1*	0.93	0.74
		ENSBTAG00000047810	*CCDC96*	0.92	0.73
		ENSBTAG00000012896	*METTL7B*	0.92	0.80
		ENSBTAG00000044101	*DGLUCY*	0.92	0.81
		ENSBTAG00000018808	*SLC25A35*	0.91	0.79
		ENSBTAG00000033642	Novel Cow gene	-0.91	-0.80
		ENSBTAG00000016912	*TSFM*	0.91	0.79
		ENSBTAG00000013587	*TXNL4A*	0.91	0.77
		ENSBTAG00000047229	*CRIP1*	0.91	0.71
		ENSBTAG00000021067	*ACTR1A*	0.90	0.83
		ENSBTAG00000047213	*GPRASP1*	-0.90	-0.77
		ENSBTAG00000009348	*OIT3*	0.90	0.82
		ENSBTAG00000017352	*ZC3H11A*	-0.90	-0.71
	Coral1	ENSBTAG00000001968	*TTC14*	0.97	-0.76
		ENSBTAG00000011889	*NOC4L*	-0.97	0.71
		ENSBTAG00000014807	*DPP8*	0.97	-0.77
		ENSBTAG00000008167	*TTC3*	0.97	-0.59
		ENSBTAG00000013651	*DNAJB12*	-0.97	0.73
		ENSBTAG00000014616	*SEPSECS*	0.96	-0.77

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000021062	*SMARCD2*	-0.96	0.66
		ENSBTAG00000011727	*ETAA1*	0.96	-0.76
		ENSBTAG00000010059	*C8H9ORF72*	0.97	-0.70
		ENSBTAG00000045601	*KCTD21*	-0.96	0.66
		ENSBTAG00000003752	*SLC25A24*	0.95	-0.73
		ENSBTAG00000031873	*ZSCAN16*	0.95	-0.76
		ENSBTAG00000007825	*MARK2*	-0.95	0.61
		ENSBTAG00000017994	*MAX*	-0.95	0.75
		ENSBTAG00000020806	*HELB*	0.95	-0.69
		ENSBTAG00000021225	*DCAF5*	-0.95	0.67
		ENSBTAG00000032331	*SLC48A1*	-0.95	0.73
		ENSBTAG00000003419	*EAPP*	0.95	-0.75
		ENSBTAG00000004718	*PUS7L*	0.94	-0.59
		ENSBTAG00000010485	*MFN1*	0.94	-0.70
		ENSBTAG00000021587	*SMPDL3A*	0.94	-0.62
		ENSBTAG00000018528	*USP45*	0.94	-0.73
		ENSBTAG00000008705	*CABLES1*	-0.94	0.79
		ENSBTAG00000018613	*NOL8*	0.94	-0.58
		ENSBTAG00000002956	*ZNF674*	0.94	-0.66
		ENSBTAG00000003035	*IMPACT*	0.94	-0.64
		ENSBTAG00000005328	*ICE2*	0.93	-0.80
		ENSBTAG00000011042	*TMEM129*	-0.93	0.67
		ENSBTAG00000019843	*HAUS6*	0.93	-0.70
		ENSBTAG00000046548	*ST6GALNAC4*	-0.93	0.64
		ENSBTAG00000005574	*CLU*	-0.93	0.69
		ENSBTAG00000034978	*FAM168B*	0.93	-0.55
		ENSBTAG00000008243	*LRRC40*	0.93	-0.84
		ENSBTAG00000009232	*PIK3CA*	0.93	-0.77
		ENSBTAG00000018937	*HMOX2*	-0.93	0.54
		ENSBTAG00000039316	*ZNF268*	0.93	-0.68
		ENSBTAG00000046612	*ZNF292*	0.93	-0.68
		ENSBTAG00000009552	*ATP2B1*	0.93	-0.59
		ENSBTAG00000020130	*CLCN3*	-0.93	0.57
		ENSBTAG00000020468	*MICU3*	0.93	-0.68

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000048206	Novel Cow gene	0.93	-0.55
		ENSBTAG00000007920	*PANK2*	0.93	-0.56
		ENSBTAG00000000533	*ZNF684*	0.93	-0.60
		ENSBTAG00000031473	*ORC2*	0.93	-0.64
		ENSBTAG00000003111	*ATM*	0.93	-0.70
		ENSBTAG00000018745	*CEP290*	0.93	-0.81
		ENSBTAG00000020761	*USP33*	0.93	-0.78
		ENSBTAG00000009822	*PPP4R1*	-0.93	0.69
		ENSBTAG00000019877	*DOCK7*	0.93	-0.72
		ENSBTAG00000001692	*NDST1*	-0.93	0.64
		ENSBTAG00000002272	*KCTD18*	0.93	-0.82
		ENSBTAG00000002378	*NUCB1*	-0.92	0.77
		ENSBTAG00000020193	*DCLRE1A*	0.62	-0.82
		ENSBTAG00000015728	*NSUN3*	0.92	-0.64
		ENSBTAG00000013275	*MAD2L2*	-0.92	0.73
		ENSBTAG00000016151	*VTN*	-0.92	0.65
		ENSBTAG00000007910	*EXOC7*	-0.92	0.83
		ENSBTAG00000003338	*LRRCC1*	0.92	-0.76
		ENSBTAG00000031377	*SYCP2L*	0.92	-0.66
		ENSBTAG00000033041	*YIPF4*	0.92	-0.71
		ENSBTAG00000020446	*THOP1*	-0.92	0.54
		ENSBTAG00000004600	*OTUD5*	-0.92	0.82
		ENSBTAG00000012485	*SPOUT1*	-0.92	0.68
		ENSBTAG00000004902	*INTS8*	0.92	-0.53
		ENSBTAG00000009565	*RASA1*	0.92	-0.87
		ENSBTAG00000008160	*MBOAT2*	0.92	-0.63
		ENSBTAG00000008576	*PLEKHD1*	-0.92	0.78
		ENSBTAG00000008997	*ENG*	-0.92	0.74
		ENSBTAG00000027728	*NUDT12*	0.92	-0.73
		ENSBTAG00000047040	*SERPINA3-3*	-0.92	0.62
		ENSBTAG00000008001	*NUCKS1*	0.91	-0.74
		ENSBTAG00000031598	*NIPSNAP2*	0.91	-0.67
		ENSBTAG00000033902	*SLC25A32*	0.91	-0.81
		ENSBTAG00000008434	*GCAT*	-0.91	0.77

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000003191	*FSCN1*	-0.91	0.55
		ENSBTAG00000014249	*IVNS1ABP*	0.91	-0.58
		ENSBTAG00000032503	*ITPRIPL2*	-0.91	0.73
		ENSBTAG00000006276	*C1QTNF1*	-0.91	0.64
		ENSBTAG00000012989	*UBE2L6*	-0.91	0.78
		ENSBTAG00000004492	*SWT1*	0.91	-0.66
		ENSBTAG00000003748	*CNOT2*	0.91	-0.64
		ENSBTAG00000003777	*TIE1*	-0.91	0.74
		ENSBTAG00000003560	*RPL8*	-0.91	0.75
		ENSBTAG00000001486	*ZNF280D*	0.91	-0.83
		ENSBTAG00000003936	*PNKD*	-0.91	0.53
		ENSBTAG00000022028	*DERL3*	-0.91	0.64
		ENSBTAG00000017095	*DENND4A*	0.91	-0.80
		ENSBTAG00000019810	*AARS*	-0.91	0.71
		ENSBTAG00000046840	*DEXI*	-0.91	0.66
		ENSBTAG00000031252	*CD82*	-0.91	0.55
		ENSBTAG00000001842	*GSTM3*	0.91	-0.52
		ENSBTAG00000011926	*ZNF808*	0.91	-0.74
		ENSBTAG00000000287	*GNPDA2*	0.91	-0.75
		ENSBTAG00000000105	*RIT1*	0.91	-0.50
		ENSBTAG00000021600	*KANSL1L*	0.91	-0.58
		ENSBTAG00000004791	*VPS26C*	-0.91	0.71
		ENSBTAG00000016516	*CYB5R3*	-0.91	0.60
		ENSBTAG00000002833	*FBXO22*	0.90	-0.61
		ENSBTAG00000000395	*ADAMTS20*	0.90	-0.58
		ENSBTAG00000032450	*PPP1R14B*	-0.90	0.70
		ENSBTAG00000040551	*ZBTB1*	0.90	-0.71
		ENSBTAG00000019748	*FAM208A*	0.90	-0.67
		ENSBTAG00000001509	*ELK3*	-0.90	0.62
		ENSBTAG00000015184	*SYDE1*	-0.90	0.75
		ENSBTAG00000017770	*C7orf55-LUC7L2*	0.90	-0.59
		ENSBTAG00000016267	*SERPING1*	-0.90	0.64
		ENSBTAG00000007130	*ESF1*	0.90	-0.70
		ENSBTAG00000017011	*CCDC125*	0.90	-0.69

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000019501	*NUDT15*	0.90	-0.81
		ENSBTAG00000005573	*SCAND1*	-0.90	0.47
		ENSBTAG00000021304	*TP53BP1*	0.90	-0.67
		ENSBTAG00000042677	*RF00273*	0.90	-0.66
		ENSBTAG00000038361	*SERPINA11*	-0.90	0.64
		ENSBTAG00000000754	*PPP2R5A*	0.90	-0.76
		ENSBTAG00000005147	*HPS5*	0.90	-0.65
		ENSBTAG00000008554	*NINJ2*	-0.90	0.82
		ENSBTAG00000024688	*PHIP*	0.90	-0.81
		ENSBTAG00000013184	*CEP120*	0.90	-0.62
	SkyBlue	ENSBTAG00000003112	*THUMPD3*	-0.98	-0.76
		ENSBTAG00000015900	*CNPY3*	0.96	0.79
		ENSBTAG00000017580	*RFX5*	0.95	0.83
		ENSBTAG00000030744	*NFYB*	-0.94	-0.75
		ENSBTAG00000003807	*CNOT9*	-0.94	-0.68
		ENSBTAG00000007578	*SHTN1*	-0.93	-0.85
		ENSBTAG00000008097	*WNT2*	0.93	0.75
		ENSBTAG00000002292	*SMARCA1*	-0.93	-0.71
		ENSBTAG00000002395	*HPS3*	-0.93	-0.79
		ENSBTAG00000007101	*F3*	-0.93	-0.72
		ENSBTAG00000016169	*ID1*	0.93	0.63
		ENSBTAG00000014101	*TSR3*	0.92	0.68
		ENSBTAG00000048287	Novel Cow gene	0.92	0.80
		ENSBTAG00000014691	*ARFGEF1*	-0.92	-0.74
		ENSBTAG00000013528	*CHMP7*	0.92	0.59
		ENSBTAG00000020634	*FBXO7*	-0.92	-0.76
		ENSBTAG00000015834	*ARHGEF4*	-0.91	-0.62
		ENSBTAG00000009417	*ZFAND5*	-0.91	-0.72
		ENSBTAG00000001005	*ZNF280B*	-0.91	-0.70
		ENSBTAG00000002126	*PFKFB2*	-0.91	-0.68
		ENSBTAG00000005728	*CDHR4*	0.91	0.71
		ENSBTAG00000002747	*ABCA5*	-0.91	-0.79
		ENSBTAG00000000382	*MED27*	0.91	0.84
		ENSBTAG00000022293	*CSKMT*	0.91	0.75

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000000632	*RAD54B*	-0.90	-0.63
		ENSBTAG00000012382	*KCTD9*	-0.90	-0.76
		ENSBTAG00000020079	*MAP3K4*	0.90	0.75
		ENSBTAG00000031950	*RAB3IP*	-0.90	-0.77
	Black	ENSBTAG00000012644	*UROD*	-0.98	0.63
		ENSBTAG00000001792	*C7H19orf70*	-0.97	0.53
		ENSBTAG00000015559	*ERI3*	-0.97	0.72
		ENSBTAG00000007809	*PPP1R36*	0.96	-0.57
		ENSBTAG00000026953	*TRIP11*	0.96	-0.68
		ENSBTAG00000016230	*BRWD3*	0.95	-0.56
		ENSBTAG00000016770	*GAPT*	0.95	-0.51
		ENSBTAG00000014399	*QSER1*	0.95	-0.55
		ENSBTAG00000011350	*CCNQ*	-0.95	0.60
		ENSBTAG00000023941	*CPN1*	-0.95	0.48
		ENSBTAG00000004874	*AURKAIP1*	-0.95	0.56
		ENSBTAG00000022777	*CDC42BPA*	0.95	-0.67
		ENSBTAG00000013126	*EMG1*	-0.94	0.54
		ENSBTAG00000011388	*STOML2*	-0.94	0.60
		ENSBTAG00000018496	*TIMM17B*	-0.94	0.56
		ENSBTAG00000018546	*LRBA*	0.94	-0.73
		ENSBTAG00000008082	*CEP350*	0.94	-0.68
		ENSBTAG00000019427	*KIAA0232*	0.94	-0.59
		ENSBTAG00000001407	*AP3S2*	-0.94	0.75
		ENSBTAG00000002948	*SETD2*	0.94	-0.66
		ENSBTAG00000047214	*TRAPPC1*	-0.94	0.53
		ENSBTAG00000005465	*NDUFA9*	-0.93	0.56
		ENSBTAG00000047734	*C16orf58*	-0.93	0.77
		ENSBTAG00000011212	*EIF3K*	-0.93	0.46
		ENSBTAG00000020958	*LRRC8D*	0.93	-0.60
		ENSBTAG00000031875	*BANF1*	-0.93	0.59
		ENSBTAG00000015466	*NSUN2*	-0.93	0.66
		ENSBTAG00000003097	*DENND4C*	0.93	-0.68
		ENSBTAG00000006125	*PLK3*	-0.93	0.60
		ENSBTAG00000046031	*C14H8orf82*	-0.93	0.74

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000013496	*CPM*	0.93	-0.52
		ENSBTAG00000000579	*NIPBL*	0.93	-0.77
		ENSBTAG00000000014	*TXN2*	-0.93	0.69
		ENSBTAG00000021093	*RPS16*	-0.93	0.65
		ENSBTAG00000002563	*WDR45*	-0.92	0.53
		ENSBTAG00000010514	*ASB7*	0.92	-0.65
		ENSBTAG00000014615	*SLC26A2*	0.92	-0.61
		ENSBTAG00000006101	*PSMD4*	-0.92	0.67
		ENSBTAG00000023867	*RC3H2*	0.92	-0.57
		ENSBTAG00000012146	*PPP1CA*	-0.92	0.69
		ENSBTAG00000002706	*GSTZ1*	-0.92	0.58
		ENSBTAG00000005652	*ALG3*	-0.92	0.82
		ENSBTAG00000014286	*IFT88*	0.92	-0.67
		ENSBTAG00000017710	*ECHS1*	-0.92	0.51
		ENSBTAG00000001783	*FBXO17*	-0.92	0.60
		ENSBTAG00000007346	*COMMD4*	-0.92	0.52
		ENSBTAG00000007900	*FIS1*	-0.92	0.74
		ENSBTAG00000004079	*ZNF106*	0.92	-0.65
		ENSBTAG00000004179	*PSMD9*	-0.91	0.50
		ENSBTAG00000019660	*TMUB2*	-0.91	0.76
		ENSBTAG00000032674	*SENP5*	0.91	-0.69
		ENSBTAG00000017809	*PDS5A*	0.91	-0.63
		ENSBTAG00000047278	*NDUFB11*	-0.91	0.73
		ENSBTAG00000019045	*COMMD9*	-0.91	0.47
		ENSBTAG00000020089	*USP32*	0.91	-0.70
		ENSBTAG00000008765	*PRADC1*	-0.91	0.53
		ENSBTAG00000019906	*VPS25*	-0.91	0.64
		ENSBTAG00000018569	*CUL4B*	0.91	-0.76
		ENSBTAG00000021288	*PSMB4*	-0.91	0.59
		ENSBTAG00000018637	*HDDC3*	-0.91	0.59
		ENSBTAG00000014777	*FBXO6*	-0.91	0.68
		ENSBTAG00000020721	*MRPL55*	-0.91	0.60
		ENSBTAG00000000147	*RIC8B*	0.91	-0.67
		ENSBTAG00000039529	*BTBD19*	-0.91	0.69

Breed-Diet	Module	Ensembl ID	Gene name	Module Membership	Gene Significance
		ENSBTAG00000018542	*COX5B*	-0.91	0.53
		ENSBTAG00000017547	*DBNL*	-0.91	0.80
		ENSBTAG00000009839	*GSTK1*	-0.90	0.59
		ENSBTAG00000017639	*RPS6KA3*	0.90	-0.63
		ENSBTAG00000019667	*RNF187*	0.90	0.67
		ENSBTAG00000046553	*SECISBP2L*	0.90	-0.53
		ENSBTAG00000012694	*UHMK1*	0.90	-0.52
		ENSBTAG00000020123	*AUP1*	-0.90	0.57
		ENSBTAG00000011583	*APEH*	-0.90	0.62
		ENSBTAG00000001855	*COQ9*	-0.90	0.81
		ENSBTAG00000009307	*RER1*	-0.90	0.77
		ENSBTAG00000000753	*PIAS4*	-0.90	0.71
		ENSBTAG00000013602	*BRWD1*	0.90	-0.70
		ENSBTAG00000019139	*PHF3*	0.90	-0.74

CH: Charolais; HF: Holstein-Frieisan; H1: High concentrate, phase 1; H2: High concentrate, phase 2.

Chapter 5.

Discussion

5. Discussion

5.1. Summary of main findings

5.1.1. Study 1

This study employed a multi-breed reference population to identify SNPs associated with FE and related traits in an Irish population of beef cattle. GWAS were carried out on a per breed basis, and summary statistics were combined using meta-analysis to identify SNPs associated with each trait for the entire population. Seven SNPs were associated with RFI, 14 with ADG and three with FI. In order to identify causal variants associated with these traits, eQTL analysis was carried out. One eQTL was identified between a SNP associated with RFI, rs43555985, and *GFRA2*.

5.1.2. Study 2

This study investigated the hepatic transcriptome of CH and HF steers divergent for RFI across three dietary phases. This study aimed to identify DEGs consistently associated with RFI regardless of breed or diet offered. Identified genes may be candidates for further investigation to develop our understanding of the biology underlying RFI and to identify potentially robust biomarkers for RFI in the form of SNPs. RNA-Seq analysis identified 355 DEGs across three dietary phases for two breeds of steers divergent for RFI. Three genes, *GADD45G*, *HP* and *MID1IP1*, were differentially expressed across two dietary phases for the CH steers but no genes were differentially expressed in more than one dietary phase for the HF steers. Similarly, no gene was differentially expressed across all three dietary phases for either breed. The lack of consistent DEGs across dietary phases within breeds supports the previous findings of Coyle et al. (2016, 2017) who observed that animals re-rank in terms of RFI when they are offered different diets or the same diet throughout their life. However, while the list of DEGs may be different, several physiological pathways were enriched for DEGs across all diet and breed combinations.

5.1.3. Study 3

This study utilised the gene expression data generated in Study 2 as the basis for a systems biology analysis to identify candidate genes associated with RFI divergence. WGCNA was carried out to identify modules of co-expressed genes. Ten modules were associated with RFI across three diet-breed combinations. Within these modules a total of 349 hub genes were identified. Thirty-seven of these hub genes were previously associated with FE, while ten were identified as differentially expressed in Study 2. Pathway analysis found that the modules associated with RFI were enriched for genes implicated in physiological processes such as the immune response, oxidative phosphorylation and protein turnover.

5.2. Strengths, weaknesses and future directions

5.2.1. Study 1

A strength of the work carried out in Chapter 2 was the utilisation of a mixed-breed reference population. Due to the breed heterogeneity of cattle present on beef production enterprises in Ireland and in other countries it has been suggested that utilisation of such reference populations may enable identification of biomarker SNPs applicable to multiple breeds (Hayes et al., 2013). Another advantage of using a reference population consisting of multiple breeds is that it may enable fine-mapping, whereby causal variants may segregate across multiple breeds (Pausch et al., 2017). eQTL analyses may facilitate the discovery of causal variants underlying the biology of the trait under study, which was RFI in this case. The eQTL identified in Chapter 2 was the first reported eQTL for a FE trait in beef cattle.

A weakness of this work is the relatively small sample size used for both the GWAS and the eQTL analysis. Kemper et al. (2016) found that increasing sample size resulted in greater accuracy of genomic prediction. Similarly, while this work captured most of the commercially relevant beef cattle breeds in Ireland, the addition of other less popular breeds to the reference population would aid in identifying markers applicable to all beef breeds present in the country.

Future work emanating from this study would primarily be the requirement to replicate the findings from Chapter 2 in other large populations of beef cattle, both in Ireland and internationally. The replication of these findings in independent populations would indicate that the identified SNPs may have utility as biomarkers (Wray et al., 2013). The replication of the eQTL observed in Chapter 2 would also provide further support that the variant implicated may be causative for variation in RFI. In addition, using SNPs identified in this study as potential biomarkers will require them not to negatively affect other economically important traits. For example, it has been suggested that animals with greater FE may have decreased fertility traits (Fontoura et al., 2015, Ferreira Júnior et al., 2018). The effect of these SNPs on fertility may be assessed by selecting cattle who display desirable genotypes at these locations with regards to RFI, and carrying out a trial to investigate their reproductive ability. Furthermore, investigation of the effects of eQTLs over time may provide insight into the biology of RFI. It may be possible that SNPs may have different effects at varying developmental stages. The identification of SNPs with consistent effect over time may both offer insight into biology while also uncovering candidate biomarkers for RFI.

As RFI, ADG and FI are complex polygenic traits, it is unlikely that the variants uncovered here will be used as individual selection markers. Rather, they could be included in equations to calculate genomic estimated breeding values (GEBVs). GEBVs are breeding values calculated by summing SNP effects for animals that have been genotyped, but do not have phenotypic data for the trait of interest (Meuwissen et al., 2016). However, the adoption of GEBVs for complex traits, such as RFI, in beef cattle has been of limited success due to the primarily crossbred nature of beef production enterprises when compared to dairy cattle (Van Eenennaam et al., 2014). It has been suggested that including SNP effects discovered in multiple-breed reference populations may improve the accuracy of GEBV equations (Khansefid et al., 2014, Meuwissen et al., 2016).

While eQTL analysis identified one SNP which may impact mRNA expression, several forms of QTL exist regulating various other molecular processes. For

example QTLs effecting splicing (Li et al., 2016), micro-RNA expression (Lappalainen et al., 2013), protein expression (Stark et al., 2014) and genome-wide methylation patterns (Hannon et al., 2015) have been identified in human studies. Identifying QTLs associated with RFI which cause variation in processes such as those mentioned here may enable further understanding of the physiology underpinning phenotypic variation in RFI.

5.2.2. Study 2

Work carried out in Chapter 3 has been the first to investigate differential gene expression in the hepatic transcriptome of two breeds of RFI-divergent beef steers across multiple dietary phases. The identification of genes that are differentially expressed across diet and breed is important to identify genes consistently associated with RFI due to the observed effect that breed and diet have on RFI (Coyle et al., 2016, Durunna et al., 2012, Crowley et al., 2010).

One potential drawback of this study was that the RFI measurement was not corrected for back-fat thickness. It has been suggested that incorporating an adjustment for this measure when calculating RFI can mitigate against the negative relationship previously observed between back-fat thickness and feed efficiency (Basarab et al., 2003, Nkrumah et al., 2004). Ensuring levels of carcass fatness is important to ensure sufficient marbling and that animals reach a minimum carcass fat score prior to slaughter (Basarab et al., 2003, McGee, 2014). As this study was part of a larger experiment which investigated the repeatability of RFI across diet, RFI was calculated without back-fat included in the RFI measurement equation (Coyle et al., 2017, Coyle et al., 2016).

Future investigations which may follow on from this work could include the transcriptomic analyses of a greater range of tissues. As well as liver, several other organs may be implicated in RFI variation such as skeletal muscle, rumen epithelium and hypothalamus (Perkins et al., 2014, Weber et al., 2016, Paradis et al., 2015). These tissues warrant study to achieve a more comprehensive insight into the biology

underpinning RFI in cattle. Investigating gene expression across breed and diet for tissues such as these may further enhance understanding of the physiological processes underlying RFI.

Additionally, the DEGs identified within this study warrant further investigation to identify SNPs within these genes that may be associated with RFI variation. This may be carried out using whole genome sequencing (WGS) which enables identification of SNPs throughout the genome (Daetwyler et al., 2014). Candidate genes, such as those identified in Chapter 3, may be prioritized for analysis to overcome the issues relating to the very large amount of data generated by WGS.

Further work to consider may be the use of dietary approaches to modulate physiological processes consistently associated with RFI divergence, such as the immune response. Antimicrobials are administered to cattle in feedlots in order to prevent the spread of infection, while also improving feed efficiency (Golder and Lean, 2016). This practice is controversial as it may contribute to antibiotic resistance and consumers are now demanding antibiotic-free meat (Cameron and McAllister, 2016, Gadde et al., 2017). However, work in broilers has demonstrated that supplementing feed with probiotics improved feed efficiency, offering a potential alternative to antibiotics (Gadde et al., 2017).

5.2.3. Study 3

Chapter 4 used systems biology approaches to identify candidate genes for biomarker discovery. Similarly to Chapter 3, a particular strength of this chapter was the investigation of RFI across dietary phases and breeds. However, in comparison to Chapter 3 the systems biology method used in Chapter 4 enables a more holistic and integrated view of the entire biological system (Zhang and Horvath, 2005). This recognises the fact that genes do not work in isolation and it is prudent to examine groups of genes which work together to affect the phenotype under study. Such investigations may identify key regulator genes and important processes implicated in divergent RFI phenotype (Langfelder and Horvath, 2008). A key strength of

Chapter 4 was the multiplicity of breeds and diets investigated. As in Chapter 3, no previous work has attempted to identify hub genes associated with RFI across multiple dietary phases for several breeds of cattle. While no hub genes were shared across multiple diets, genes previously associated with RFI were identified as hub genes within this study. These are interesting candidate genes for future work to uncover SNPs associated with RFI. Employing targeted re-sequencing of these candidate genes may identify SNPs of interest with utility as biomarkers for RFI (Mullen et al., 2012).

A potential weakness to this study is the relatively small sample size. While the authors of WGCNA recommend a minimum sample size of ten to fifteen samples (Langfelder and Horvath, 2008, Langfelder, 2017), a sample of greater than 20 has been suggested for generating networks with highest connectivity (Ballouz et al., 2015). With an increased sample size, it may be possible to carry out consensus module analysis. This type of gene co-expression experiment investigates module expression across diet, breed or tissue for example (Langfelder and Horvath, 2007).

5.3. Insight into mechanisms underlying RFI variation

5.3.1. The immune response

Variation in the immune response has been postulated as one potential physiological function underlying variation in RFI. For example, cattle with high-RFI may be experiencing chronic inflammation and consequently partition less energy to growth as the immune system is an energetically costly physiological process (Alexandre et al., 2015). However, other studies have suggested that low-RFI cattle display increased levels of immune related gene expression, and consequently they respond to infection in a more energy efficient manner than high-RFI cattle (Paradis et al., 2015, Weber et al., 2016). Similarly in pigs, it has been observed that low-RFI animals exhibited decreased expression levels of immune related genes following an exogenous immune challenge, suggesting that feed efficient animals expend less energy fighting infection than their inefficient counterparts (Vigors et al., 2016).

Results from the work carried out as part of this thesis further support the hypothesis that the immune response is central in RFI divergence. For example in Chapter 2, SNPs nearby to two genes with immune functionality were associated with variation in ADG, a component trait of RFI. This is in agreement with previous work whereby SNPs associated with FE and related traits have been found close to genes implicated in immune function (de Oliveira et al., 2014). In Chapter 3, gene set enrichment analysis of DEGs found that pathways implicated in the immune response were significantly enriched across all dietary phases for both breeds investigated as part of this work. Similarly, two genes identified as differentially expressed in two dietary phases for the CH steers, *HP* and *GADD45G*, function in the immune response. Using a systems biology approach in Chapter 4, two hub genes, *CFD* and *RFX5*, have roles in the immune system. Both of these genes have previously been associated with RFI. *CFD*, which encodes for a complement protein was identified as differentially expressed in the hepatic transcriptome of RFI-divergent Nellore steers (Tizioto et al., 2015). *RFX5*, which is an important transcription regulator of immune genes, has been observed to be a DEG in the liver of RFI-divergent dairy cows (Salleh et al., 2017). As hub genes are suggested to be highly connected regulators of important biological functions, these genes may be candidates for further interrogation to discover SNP biomarkers for RFI (Langfelder and Horvath, 2008). Several modules across both breeds under study were also found to be enriched for genes implicated in physiological functions related to the immune system. Furthermore, the results reported in thesis may indicate that increased basal activity of the immune system may lead to less desirable feed efficiency. It is possible that increased activation of the immune system may cause cattle to partition more consumed energy to maintenance of this physiology response rather than growth. This would cause poorer feed efficiency.

5.3.2. Lipid metabolism

Lipid metabolism and the deposition of lipid as adipose tissue is an energetically costly process (Lawrence et al., 2012b). It has been hypothesized that inefficient cattle partition more energy towards fat deposition rather than muscle synthesis (Berry and Crowley, 2013). Several transcriptomic studies across tissues have identified genes implicated in lipid metabolism as differentially expressed between

RFI-divergent cattle (Mukiibi et al., 2018, Tizioto et al., 2015, Tizioto et al., 2016, Weber et al., 2016, Salleh et al., 2017). Mukiibi et al. (2018) suggested that low-RFI cattle are likely to expend less energy on the deposition of lipid than high-RFI cattle and consequently require less energy to accumulate muscle. The results reported in Chapter 3 support this hypothesis. *MID1IP1*, a gene required for lipid synthesis, was observed to be downregulated in the low-RFI CH steers offered a high-concentrate diet and a zero-grazed grass diet (Kim et al., 2010). Previous work in Canadian beef steers also observed downregulation of this gene in low-RFI cattle (Mukiibi et al., 2018). Several DEGs identified in Chapter 3 were also identified previously as differentially expressed in RFI-divergent cattle and had functions pertaining to lipid metabolism. For example, *IRS2* is a gene upregulated in the low-RFI steers offered a high-concentrate diet and was previously shown to have increased expression in feed efficient pigs (Gondret et al., 2017). Pathway analysis of DEGs identified in Chapter 3 also highlighted the enrichment of the lipid metabolism physiological process across all breed-diet combinations. The consistent enrichment of this physiological function across all diets and breeds indicates that it is important with regards to RFI-divergence regardless of environmental influences. Identifying pathways associated with RFI consistently is important to facilitate the discovery of robust biomarkers for RFI applicable across a range of diets and breeds of cattle. Work carried out in Chapter 4 identified two hub genes, *NSUN2* and *NIPBL*, implicated in lipid metabolism and which have both been previously associated with FE. *NSUN2* was reported to be nearby to a SNP associated with RFI (Serão et al. 2013b), while *NIPBL* is within a quantitative trait loci for RFI identified in broilers (Liu et al., 2018a).

5.3.3. Oxidative phosphorylation and mitochondrial efficiency

Mitochondrial efficiency and oxidative phosphorylation have previously been shown to vary between high- and low-RFI animals. Work in broilers has demonstrated that feed inefficient broilers have decreased energy production compared to their efficient counterparts due to proton leakage across the mitochondrial membrane (Bottje and Carstens, 2009). The importance of energetic efficiency was further illustrated by work reported in Chapter 4 of this thesis. Modules associated with RFI across each diet-breed combination were enriched for genes implicated in processes such as

mitochondrial dysfunction and mitochondrial energy production. Furthermore, two hub gene identified in Chapter 4, *NDUFB11* and *COX5B*, play roles in energy production within the mitochondria. *COX5B* is of particular interest as it encodes for a protein which is part of the mitochondrial respiratory complex (Stelzer et al., 2016). Several other studies have identified members of this complex to be dysregulated in FE-divergent animals (Kelly et al., 2011, Lassiter et al., 2006). Due to the importance of this complex in energy production, further investigation is warranted to fully elucidate its role in RFI-divergence. Similarly, a previous transcriptomic study observed upregulation of genes involved in energy production in the rumen epithelium of low-RFI cattle, while also observing that the number of mitochondria were fewer (Kong et al., 2016b). This potentially indicates that the mitochondria of efficient cattle produce more ATP than those of inefficient cattle.

As well as energetic efficiency, the production of reactive oxygen species (ROS) has been observed to be differential in FE-divergent animals. Work carried out in pigs observed that low-RFI pigs generated less ROS than high-RFI pigs (Grubbs et al., 2013). Similar findings have been reported for broilers (Bottje and Carstens, 2009). In cattle, increased expression of genes which encode for protein that detoxify ROS has been reported in high-RFI steers (Kelly et al., 2011). Work carried out in Chapter 3 identified a gene, *GSTM1*, which functions in the response to oxidative stress as differentially expressed in HF steers offered a high concentrate diet. This gene was previously observed to be differentially expressed in RFI-divergent cattle in several studies (Chen et al., 2011, Paradis et al., 2015, Tizioto et al., 2015). Similarly, WGCNA carried out in Chapter 4 identified hub genes and enriched biological pathways associated with oxidative stress. For example, *GSTM3*, an isoform of *GSTM1*, was classified as a hub gene in a module identified for the HF steers offered a high concentrate diet. While pathway analysis of genes within modules associated with RFI highlighted enrichment of genes associated with oxidative phosphorylation and production of ROS.

5.3.4. Protein metabolism

Protein turnover and metabolism is an energetically costly process, which has previously been suggested as a mechanism underlying a proportion of variation in RFI (Cantalapiedra-Hijar et al., 2018). Ramayo-Caldas et al. (2018), carrying out gene network analyses on RFI-divergent pigs, observed that pathways associated with protein metabolism were upregulated in low-FE pigs. Studies in cattle also noted that genes implicated in protein synthesis and degradation to be differentially expressed between high- and low-RFI steers (Kong et al., 2016b, Chen et al., 2011). Similarly, results from the GWAS meta-analyses carried out in Chapter 2 identified SNPs nearby to or within genes associated with protein metabolism. For example, rs41638273 which was associated with variation in RFI is located within a quantitative trait locus (QTL) associated with *MSTN* (Saatchi et al., 2014b). Mutations in the *MSTN* gene are responsible for variation in muscle morphology (Grobet et al., 1997). rs382426807 was associated with ADG via GWAS in Chapter 2. This SNP is located within an exon of *STAT5A*, which is an important gene associated with muscle growth. These results from the GWAS carried out in Chapter 2 further support the hypothesis that variation in protein metabolism may be a physiological process implicated in RFI divergence. Interestingly, WGCNA carried out in Chapter 4 also suggests a role for protein turnover in RFI-divergence. Hub genes within modules in both CH and HF steers offered a high concentrate diet were associated with protein turnover and degradation, while pathways within a module for the CH steers were enriched for genes associated with protein degradation. These results taken with previously reported results in the literature support the role of protein turnover in RFI-divergence. The SNPs associated with RFI and ADG in Chapter 2 may be candidate biomarkers for these traits, however, replication would be required in independent studies to validate these associations. In addition, the hub genes and pathways associated with this trait in Chapter 4 warrant further investigation to uncover SNPs associated with RFI and to elucidate the biological role of these genes and pathways related to variation in RFI.

5.4. Potential biomarkers for RFI

A major obstacle to the incorporation of RFI as a selection tool is the difficulty and expense associated with its measurement. In order for RFI to be calculated, an animal's

individual FI and ADG must be measured over a period of 70 days (Cundiff et al., 2010). This is not possible at the level of individual beef producers. The discovery and validation of biomarker SNPs for inclusion on the International Dairy and Beef (IDB) chip, which is a custom genotyping panel developed for Irish farmers (Mullen et al., 2013), would remove the requirement for continual and expensive feeding trials. Biomarker discovery would enable producers to rapidly identify FE cattle, improving profits and reducing the environmental impact of beef production (Fitzsimons et al., 2017). However, the identification of robust biomarkers for RFI is complicated by the observed re-ranking of cattle when offered varying diets throughout their lifetime and the differences in RFI observed between breeds (Durunna et al., 2012, Coyle et al., 2016, Crowley et al., 2010). Any putative biomarker must be robust and consistent regardless of diet offered or breed of cattle under test. Work carried out within this thesis aimed to identify such biomarkers.

In Chapter 2, due to the multi-breed and crossbred nature of the beef herd in Ireland (Wickham et al., 2012), individual breed GWAS summary statistics were combined via meta-analysis to identify SNPs which may be applicable as multi-breed biomarkers. All SNPs identified as associated with RFI and related traits as part of this study warrant further investigation and require replication and validation in large populations of beef cattle prior to their inclusion in genomic assisted breeding programmes. Furthermore, in Chapter 2 a subset of SNPs previously associated with RFI were tested for within in study to assess their potential as biomarkers for RFI in Irish cattle. None of these SNPs reached genome-wide significance, highlighting the difficulty in identifying robust biomarkers for RFI, particularly across breeds.

While Chapter 2 addressed the multi-breed and crossbred nature of beef production, this GWAS did not account for the difficulties associated with identifying biomarkers for RFI across multiple dietary phases. Chapters 3 and 4 had the aim of identifying candidate genes for biomarker discovery across breed and diet offered. In Chapter 3, DEGs were identified for steers across different diet types throughout their lifetime using RNA-Seq. While no gene was differentially expressed across all dietary phases for either breed, three genes were differentially expressed in the CH steers across two

dietary phases. Two of these genes, *HP* and *MID1IP1*, displayed consistent direction of effect across both phases for which they were differentially expressed. In agreement with findings in Chapter 3, *MID1IP1* was previously observed to be downregulated in low-RFI steers (Mukiibi et al., 2018). As this gene has been associated with RFI consistently across diets and multiple breeds, it is an interesting candidate gene for the discovery of biomarkers associated with RFI.

HP which was observed to be upregulated in low-RFI CH steers in Chapter 3, was previously noted to have decreased levels of expression in low-RFI Canadian Angus steers (Mukiibi et al., 2018). Conflicting directions of expression were also noted in Chapter 3 for *GADD45G* which displayed increased levels of expression in low-RFI CH steers offered a high-concentrate diet, but decreased expression in low-RFI CH steers offered a zero-grazed grass diet. Interestingly, *GADD45G* was recently identified as a hub gene within a module associated with RFI (Sun et al., 2018). The association of this gene with RFI across multiple studies highlights it as a promising candidate gene for further investigation. Similarly, *GSTM1* was identified as downregulated in low-RFI HF steers offered a high-concentrate diet, which is in agreement with previous work (Chen et al., 2011). However, this same gene has been observed to be upregulated in low-RFI cattle previously (Paradis et al., 2015, Tizioto et al., 2015). This variation in expression of these two genes highlights the difficulties in identifying candidate genes for RFI biomarker discovery and the importance of replicating findings in multiple breeds and dietary phases. Despite these inconsistencies regarding direction of expression in FE cattle, both of these genes are candidates for further work to elucidate their role in the biology of RFI.

WGCNA carried out in Chapter 4 enables identification of hub genes, which may be key in controlling complex physiological processes such as those that likely underpin RFI (Suravajhala et al., 2016). Therefore, knowledge of such genes may enable discovery of biomarkers with use as selection tools for traits of interest (Zotenko et al., 2008, van Dam et al., 2018). Hub genes identified by this study, and associated with FE previously, may be ideal candidates for further study to uncover biomarkers associated with RFI.

5.5. Concluding remarks

Due to its moderate heritability and association with decreased methane emissions, RFI is an attractive trait for selection of FE cattle (Fitzsimons, et al., 2013). However, prior to the incorporation of this trait in genomic assisted breeding programmes, associated SNPs must be uncovered and validated. Additionally, the biology underlying RFI is complex and multifaceted, further understanding of the processes underlying this trait is also required. Work carried out within this thesis aimed to both elucidate the biology underpinning RFI variation and to identify SNPs and candidate genes for selection of low-RFI (FE) cattle. SNPs uncovered in ongoing work, aiming to identify variants associated with low-RFI status and low methane emissions, may also enable selection of efficient cattle with reduced environmental impact. Results reported within this thesis offer insight into the biology underpinning RFI. The SNPs and eQTL identified as associated with RFI from Chapter 2 represent potential biomarkers for RFI, following validation in independent populations. The genes and pathways identified in Chapters 3 and 4 as associated with RFI-divergence also have use as candidate genes for discovery of biomarkers. The results reported here have utility as independent populations within which other researchers may replicate their findings. This is important as finding genes or SNPs associated with RFI across multiple diets, breeds and populations may aid in prioritising candidate biomarkers. However, it is important to note that the internationally identified SNPs tested for in Chapter 2 did not replicate in this population of cattle and that no DEGs were present in all breed-diet combinations in Chapter 3. The difficulty in replicating and validating findings highlight the difficulty in identifying robust biomarkers for RFI across diet, breed and population of cattle and further suggest that genotype-by-environment interactions may play a major role in the RFI-divergence in beef cattle.

Chapter 6

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6. Bibliography

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Appendices

Appendix A: Publications arising from this thesis

1. **Higgins M.G**., Fitzsimons C., McClure M.C., McKenna C., Conroy S., Kenny D.A., McGee M., Waters S.M., Morris D.W. (2018) GWAS and eQTL analysis identifies a SNP associated with both residual feed intake and *GFRA2* expression in beef cattle. Scientific Reports 8, 14301 (2018). https://doi.org/10.1038/s41598-018-32374-6

Appendix B: List of presentations

Oral presentations

- American Society of Animal Science, Vancouver Canada, July 2018.
- British Society of Animal Science, Dublin Ireland, April 2018.
- Galway Threesis Heats and Final, Galway Ireland, November 2016.

Poster Presentations

- American Society of Animal Science, Vancouver Canada, July 2018.
- 6th NUIG-UL Postgraduate Research Day, Limerick Ireland, April 2016.