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A search for quantitative trait loci involved in physiological processes related to milk production in dairy cattle

A thesis presented in partial fulfilment of the requirements

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Abstract

Metabolic challenges have previously been used to identify physiological markers to assist with the selection of both sexes dairy cattle at an early age to increase rate of genetic gain. Physiological markers have not been implemented in selection programmes due to low accuracy. An experiment was undertaken to investigate the use of metabolic quantitative trait loci (QTL) for improving the rate of genetic gain in dairy cattle. Three metabolic challenges (adrenaline, glucose, and thyrotropin-releasing hormone) were conducted on 882 18-month-old Friesian-Jersey F_2 crossbred heifers. An initial whole genome scan was conducted by genotyping 1679 animals within the trial pedigree for 283 microsatellite markers, obtained primarily from published marker maps. QTL analyses were performed on the Friesian-Jersey crossbred trial data using metabolic and milk production phenotypes. 581 QTL were significant at the 1% level and 275 of them were QTL of metabolic phenotypes. An objective of this study was to identify chromosomal regions in which endocrine and milk production QTL were co-located, in the hope that these regions would contain genes with a significant impact on the control of milk production. The region selected for a candidate gene study was 47-51 cM of BTA14 due to the close proximity of metabolic and milk production QTL co-located in this region. Comparative mapping was used to generate a list of 105 genes in the region of interest. The genes considered the most suitable candidates for the QTL in the region were tripartite motif-containing 55, ubiquitin-conjugating enzyme E2W (putative), nuclear receptor coactivator 2, serum/glucocorticoid regulated kinase family, member 3, opioid receptor, kappa 1, proenkephalin, corticotropin releasing hormone. A major finding of this study was that there were very few chromosomal regions in which metabolic and milk production QTL were co-located. This is likely to be due to the highly complex and integrated molecular networks controlled by many genes that influence milk production traits. The data generated in this thesis will be suitable for more advanced examinations of the genetic control of milk production using the new generations of single nucleotide polymorphism chips.

Dedicated to my mother Ellen Hutchinson,

who always nurtured my love of learning.

For you my love and gratitude knows no bounds.

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Love doesn't make the world go round; love is what makes the ride worthwhile.

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List of Abbreviations

ALS	Acid labile subunit
ARF	ADP-ribosylation factor
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
AUC	Area under the (response) curve
BCS	Body condition score
BTA	*Bos taurus* autosome
BI	Breeding index
cAMP	Cyclic adenosine-3',5'-monophosphate
CNS	Central nervous system
COP	Coat protein complex
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
EB	Energy balance
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum-Golgi intermediate complex
FAS	Fatty acid synthetase
FFA	Free fatty acids
FJXB	Friesian-Jersey crossbred
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide-exchange factor
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GLUT	Solute carrier family 2 (facilitated glucose transporter)
GM	Genetic merit
GPCR	7-membrane-spanning G-protein coupled receptor
GTP	Guanosine-5'-triphosphate
HBI	High breeding index
HGM	High genetic merit
HPA	Hypothalamo-pituitary-adrenal
ICV	Intracerebroventricular
IGF-I	Insulin-like growth factor I

IGFBP	Insulin-like growth factor binding protein
LBI	Low breeding index
LIC	Livestock Improvement Corporation
LPL	Lipoprotein lipase
MAS	Marker assisted selection
ME	Metabolisable energy
MEC	Mammary epithelial cell
NR	Nuclear receptor
PC	Pyruvate carboxylase
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
mRNA	Messenger ribonucleic acid
NEFA	Non-esterified fatty acids
NPY	Neuropeptide Y
NZ	New Zealand
QTL	Quantitative trait loci/locus
rRNA	Ribosomal ribonucleic acid
TIDA	Tuberoinfundibular dopamine
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
UPP	Ubiquitin-proteasome pathway
VFAs	Volatile fatty acids
VMH	Ventromedial nuclei
3-MH	3-methyl histidine

CHAPTER 1

Introduction

Lactation and female reproduction are the principal traits of economic importance to the dairy industry. After many years of selective breeding there is no evidence to suggest that genetic variation is reducing in traits under selection pressure in out-bred dairy cattle populations (Pryce *et al.* 2007). Hence, there is still considerable potential for future genetic improvement in traits of economic importance. The focus of the research described in this thesis was to investigate ways to improve the capture of this genetic potential and in turn increase the rate of genetic gain in dairy cattle.

Genetic progress in milk production of dairy cattle is hampered because milk yield is a sex-limited trait, expressed only in the lactating cow. This is a particular limitation for sire selection, as the sire contributes half of the genes to the next generation, but genetic merit for milk production cannot be measured directly. Progeny testing to evaluate the performance of daughters is necessary to obtain accurate estimates of the bulls' genetic merit for traits such as milk production. This allows the extensive use of genetically superior bulls via mating using artificial insemination. Although progeny testing schemes currently are the best and most accurate method of selecting bulls, they have the associated disadvantage of increasing the generation interval and therefore slow the annual rate of genetic change. Genetic progress could be increased considerably if the genetic merit of potential sires could be predicted directly early in life.

The onset of copious milk synthesis and secretion is accompanied by changes in hormone concentrations as well as tissue sensitivity and responsiveness to these hormones, which co-ordinate changes in physiology that partition nutrients towards milk production. Differences in gene expression and/or allele variants underlie the metabolic pathways involved in milk production. Therefore, the rate-limiting steps that influence milk production will be observable in both sexes. Selection of animals using a physiological marker or a genetic marker (a segment of DNA with an identifiable physical location on a chromosome, whose inheritance can be followed and is associated with a phenotype) for a milk production trait could improve the

current rates of genetic gain through increased selection pressure in both dam and sire selection as well selection of replacement heifers.

A metabolic challenge broadly involves administration of an exogenous hormone or metabolite into the circulation followed by collection of a series of blood samples over time to assess the induction of hormone or metabolite release. Various challenges have been used in an attempt to find reliable physiological markers of merit for milk yield that could be measured in calves of both sexes to identify those animals of superior genetic merit. Selection of calves rather than adult animals would shorten the generation interval and accelerate the rate of genetic gain. Differences in hormone or metabolite concentrations have been found among animals that differed in genetic merit in some experiments, however, results among experiments were often inconsistent. Very few studies have been reported in which animal selection was based on physiological markers. Experiments that have been reported show that selection based on a physiological marker does not improve the correlated production traits. Thus, attempts to identify physiological markers that could be used to select high genetic merit animals of both sexes, for milk production traits at an early age were not successful.

The work described in this thesis focuses on a number of parameters that describe the essential features of hormone and substrate responses to exogenous hormone or metabolite challenges. It is anticipated that these responses will be the most important aspects of metabolic pathways involved in milk production. These parameters will be referred to as "metabolic phenotypes" and were used to search for QTL. Thus, the current study combined the previously used challenge methodology with the more recent QTL mapping technology. The intention was to discover QTL that are associated with genes underlying some of the important metabolic pathways involved in milk production. The overall aim of this thesis was to explore the use of metabolic phenotypes as a means of improving the rate of genetic gain in dairy cattle.

The objectives of the work presented in this thesis were to:

1. Describe the current breeding schemes being used for genetic improvement in the dairy industry and how the use of marker assisted selection through the identification of quantitative trait loci could impact on the current rates of genetic improvement. Provide an overview of the major metabolic pathways involved in lactation in the dairy cow. Describe the metabolic and hormonal challenges that have been used in previous research, how they provide potential physiological indicators of milk production and discuss the reasons for selecting the challenges used in the current work (Chapter 2).

2. Define the metabolic phenotypes for the variables measured in response to each challenge and discuss the reason for selection of these phenotypes. Discuss the possible impact of environmental variables and present the models used for adjusting the data (Chapter 4).

3. Define the process employed to map QTL using the metabolic phenotypes generated from the endocrine and milk production phenotypes. Select a chromosomal region to investigate and generate a list of candidate genes (Chapter 5).

4. Identify the process used to investigate each gene in the candidate gene list. Integrate the information to conclude which is most likely candidate gene and how it may be involved in the genetic control mechanisms of the QTL (Chapter 6).

5. Determine the implications of the findings of this thesis, the usefulness of metabolic phenotypes for the detection of QTL for milk production the direction of future research (Chapter 7).

CHAPTER 2

Literature Review

2.1 Introduction

The principal characteristics of economic importance to the dairy industry are lactation and female reproduction (Garrick and Snell 2005). Despite many years of selection and breeding, there is still large variation in the ability of individual cows to produce milk. In a study of 317 cows over 3 lactations, more than 72% of the variability in energy-corrected milk yield was due to between cow differences (Ingvartsen and Friggens 2005). Without the creation of new variation by mutation, response to selection can not continue indefinitely and a selection limit will be reached when the genes segregating in the population are brought to fixation. A selection limit is reached in about 10 generations in selection experiments in inbred populations (Falconer and Mackay 1996). After many years of selective breeding, there is no evidence to suggest that genetic variation is reducing in traits under selection pressure in out-bred dairy cattle populations (Pryce *et al.* 2007). Rates of genetic gain in domestic livestock species expressed as percentages of the means, are mostly in the range from 1 to 2% per year (Falconer and Mackay 1996). Hence, there is considerable potential for increasing the population mean for traits of economic importance. This has led to the development of various breeding schemes to exploit this potential, but these schemes have limitations. The objective of this thesis was to explore the use of metabolic phenotypes as a means of improving the rate of genetic gain in dairy cattle in New Zealand.

2.2 Breeding schemes

Genetic progress in milk production of dairy cattle is limited because milk yield is a sex-limited trait, expressed only in the lactating cow (Sejrsen and Løvendahl 1986, Sinnett-Smith *et al.* 1987). This is a particular limitation for sire selection, as the sire contributes half of the genes to the next generation, but genetic merit for milk production cannot be measured directly (Garrick *et al.* 1993, Sejrsen and Løvendahl 1986).

In the New Zealand dairy industry replacement sires are generated using artificial insemination to mate the highest ranking sires with the elite females that remain in the ownership of

individual farmers (Garrick and Snell 2005). These are the sire-to-breed sire (SS) and dam-to-breed sire (DS) pathways (Figure 2.1). The estimated genetic merit of the resulting male offspring is the average merit of their parents, however, some bulls will have inherited a sample of genes from their parents that confer above average genetic merit, as a result of Mendelian Sampling, while others will have inherited a below average sample. Therefore, progeny testing to evaluate the performance of their daughters is necessary to obtain a reliable assessment of each bull's genetic merit (Garrick and Snell 2005). While this strategy provides an accurate prediction of the young bull's genetic merit, it results in a long generation interval which in turn reduces the rate of genetic gain. This limitation could be overcome and genetic progress increased considerably if the genetic merit of potential sires could be predicted directly in the bull itself early in life (Sejrsen *et al.* 1984). Furthermore, the genetic merit of replacement heifers, the dam-to-breed dam (DD) and sire-to-breed dam (SD) pathways (Figure 2.1), is not accurately reflected by their production records as mature cows (Garrick and Snell 2005). There is potential to enhance the accuracy of the cow genetic merit estimates.

The introduction of herd testing and progeny testing, along with advances in semen technology have all had a major impact on the improvement in rates of genetic gain that have occurred in the past 50-60 years (Baker *et al.* 1990, Davis 2005). Significant improvements in the rate of genetic gain, under current breeding schemes, will only result if there are major changes in the structure of the industry, technology or economics (Garrick and Snell 2005). Thus, a new technology could be included in the selection programme if the economic benefit from increased genetic gain outweighed the cost of the new technology.

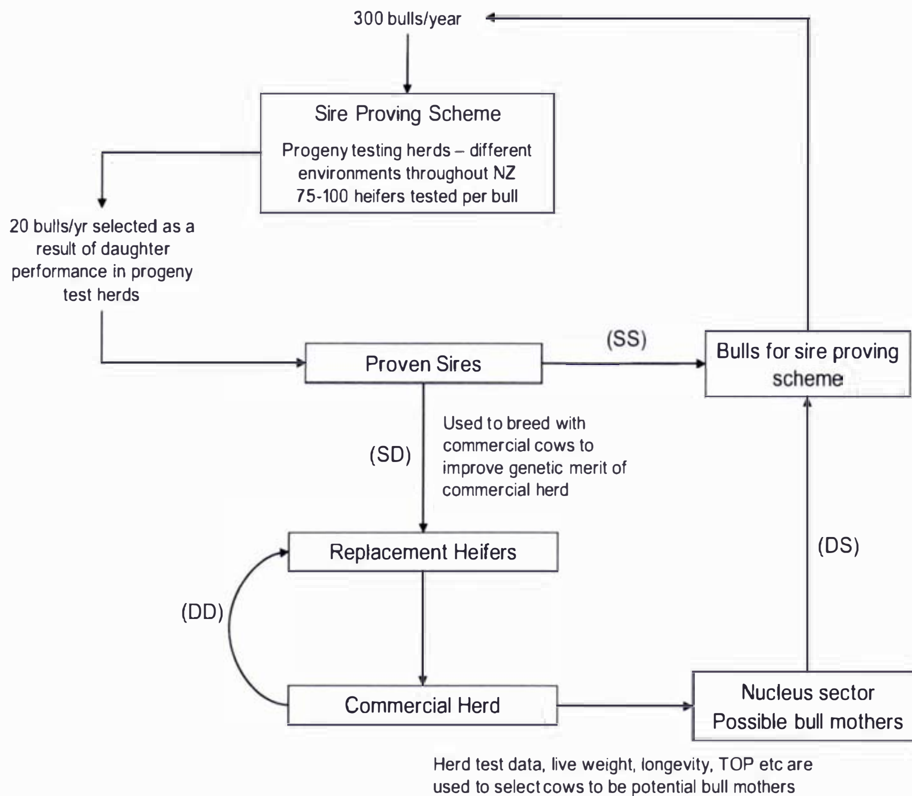


Figure 2.1 Example of the structure of a dairy cattle breeding scheme in New Zealand, based on the structure used by Livestock Improvement Corporation Limited.

Selection of animals using a physiological marker or a genetic marker (a segment of DNA with an identifiable physical location on a chromosome, whose inheritance can be followed and is associated with a phenotype) for a milk production trait could improve the current rates of genetic gain through all four selection pathways (Figure 2.1). This improvement requires that the marker either increases the accuracy of the genetic merit and/or increases the selection intensity and/or decreases the generation interval. A greater number of bulls could undergo screening for breeding values (BV – estimated genetic merit (Blair and Garrick 2007)) using physiological or genetic markers, with only the more promising ones being retained to enter progeny testing schemes (Robinson *et al.* 1992). Selection of dams to breed sires could also be improved by using physiological or genetic markers as an additional level of selection prior to selecting the dams based on their BVs predicted from milking and pedigree records.

2.3 Quantitative Trait Loci and Marker Assisted Selection

A quantitative trait locus (QTL) is defined as a chromosomal region with a Mendelian transmission pattern and with an effect on the trait of interest (Boichard *et al.* 2003, Schrooten *et al.* 2005). The major objective of QTL studies is to identify linkage between genetic markers and specific QTL that can be used in breeding programs through marker assisted selection (MAS) (Khatkar *et al.* 2004, Schrooten *et al.* 2005). If genetic markers control a significant proportion of the genetic variation in a specific quantitative trait this may enable more precise prediction of an animal's BV than collecting phenotypic information or may be added to the phenotypic information (Khatkar *et al.* 2004, Stam 1986).

The potential of MAS to increase the rate of genetic gain is dependent on QTL size and frequency in the bull population (Spelman and Garrick 1997, Stam 1986). Marker assisted selection could be particularly valuable in dairy cattle because most traits of interest are sex limited, the generation interval is long (the widespread use of a progeny test bull occurs when accurate estimates of BV are available by which time the bull is 5 years and 2 months of age (Spelman and Garrick 1997)), and progeny testing is costly (Druet *et al.* 2006). Effective MAS would enable screening and pre-selection of a larger number of young candidate bull calves increasing the selection intensity of bulls entering the progeny testing scheme or MAS could be used in a two stage selection process where pedigree records, MAS and progeny testing could all be used to increase accuracy, thus increasing genetic gain (Khatkar *et al.* 2004, Mackinnon and Georges 1998).

Despite their potential, there are no published examples of QTL selection improving national rates of genetic gain and out-competing traditional selection approaches in dairy cattle (Garrick and Snell 2005). Marker alleles surrounding a favourable QTL allele may be in a different alignment to the marker alleles that surround the favourable allele in another family. This makes within-family studies necessary to identify the favourable marker alleles surrounding a known QTL. An exception being, when the markers are in linkage disequilibrium, which occurs

when the QTL allele is a recent occurrence in the population so the same marker alleles may be useable across families (Garrick and Snell 2005, Liu and Dekkers 1998, Mackinnon and Georges 1998). The second reason that QTL selection has not been shown to improve rates of genetic over and above traditional selection approaches is that currently, known QTL only influence a small proportion, less than 15%, of the genetic variation associated with the production trait (Cowan *et al.* 1990, Garrick and Snell 2005, Spelman *et al.* 1999), thus do not provide greater accuracy than the currently used progeny testing schemes.

Genetic variation will involve a small number of major genes, a larger number of genes with moderate effects, and a very large number of genes with minor effects (Liu and Dekkers 1998). The infinitesimal genetic model, which assumes a large number of genes each contributing a small fraction of the total genetic variation in a trait (polygenes), forms the basis of quantitative genetic theory and is used to generate breeding values used in animal selection schemes. (Garrick and Snell 2005, Liu and Dekkers 1998). However, major genes (genetic variation due to segregation of alleles at a single locus) have been reported in a number of species such as the Booroola fecundity gene in merino sheep (Davis and Kelly 1983) and the *ob* (leptin) gene which is a recessive single-gene obesity mutation in mice (Houseknecht *et al.* 1998). Major genes contributing very large effects may already be fixed in elite populations, although variability might be detectable between breeds selected for different production traits (Cowan *et al.* 1990). If genes contributing very large effects are not fixed or at high frequency, caution is needed as they may have deleterious pleiotropic effects or be tightly linked to other genes with detrimental effects (Spelman and Garrick 1997). For example, a major gene for milk and fat yield was found to be tightly linked to a recessive lethal genetic disorder (bovine progressive degenerative myeloencephalopathy) in Brown Swiss (Hoeschele 1990).

Only a few genetic markers or genes that regulate milk production have been identified to date (Davis 2005), which is a major factor limiting widespread use of DNA tests (e.g. microsatelites or single nucleotide polymorphisms (SNPs) (Blair and Garrick 2007)) in genetic evaluation schemes. However, research over the past two decades has identified many bovine

chromosomal regions that are associated with variation in milk production, composition and other traits in dairy cattle (Davis 2005) and can be viewed in integrated databases of bovine QTL such as that described by Polineni *et al.* (2006) accessed at http://bovineqtl.tamu.edu. A large scale project involving the collection of milk production and physiological phenotypic data will likely lead to the discovery of novel QTL, and possibly genes, that would contribute to the current knowledge of the bovine genome and make the use of genetic markers in genetic evaluation schemes possible. Once QTL have been discovered, and candidate genes identified using bioinformatics tools, biochemical function can then be investigated and this information used to look for the gene responsible for the QTL. In addition to being useful for selection schemes, identification of single alleles linked to differences in performance could yield insights into the biochemical control of lactation (Cowan *et al.* 1990).

Metabolic pathways involved in milk production and the control of these pathways will be discussed in the remainder of this review of literature. Differences in gene expression and/or allele variants will underlie these pathways, and their rate-limiting steps, and will influence milk production. The following sections contain a review of the current scope and depth of understanding of the important metabolic pathways of nutrient partitioning particularly during lactation. The discussion in the following sections focuses on metabolic pathways in lactating ruminants, however, the experiments conducted for the current study assessed traits in young non-lactating animals. The objective of the following sections is to develop the theoretical framework for a systematic search for QTL, which is first step in identifying genetic markers or the actual genes for traits of interest.

2.4 The Major Metabolic Pathways in the Lactating Dairy Cow

Lactation greatly increases nutrient requirements of the dairy cow (Vernon 1989). At peak lactation, the energy requirements for milk synthesis can be up to 80% of a cow's net energy intake (Collier 1985). Genetic selection programmes based solely on increased milk production have resulted in cows that are genetically predisposed to a greater degree of negative energy

balance (NEB) in early lactation. The NEB occurs because increased dry matter intake only accounts for about half of the milk yield response to selection (Veerkamp 1998, Veerkamp and Koenen 1999). Thus, high producing dairy cows go into negative energy balance after calving, because dietary supply of nutrients is insufficient to meet the nutrient requirements for milk synthesis and maintenance (Bell 1995, de Vries and Veerkamp 2000, Van den Top *et al.* 2005). This necessitates substantial mobilisation of body reserves to support milk production (Vernon 1998, Vernon and Pond 1997), which is in part facilitated by altered concentrations of metabolic regulatory hormones such as insulin and GH (Bonczek *et al.* 1988, Sartin *et al.* 1988). It has been reported that for the first third of lactation, concentrate fed cows were in negative energy balance and used body energy reserves to meet their nutrient requirements (Bauman and Currie 1980). Use of ^{13}C as a marker revealed that 43-54% of the C in milk fat in early lactation, forage fed, cows was from body reserves (Wilson *et al.* 1988).

A change in physiological state alters the serum concentrations of chronically-acting hormones that have tissue specific effects on both the amounts and activity of metabolic enzymes (influencing hormonal sensitivity and responsiveness) and also the proteins which regulate them (Rhoads *et al.* 2004, Vernon 1998). These effects of chronically-acting hormones are through, both, changes in gene expression and changes in intracellular signalling systems, thus elicit homeorhetic adaptations (Vernon 1998). The metabolic adaptations that occur during lactogenesis are homeorhetic mechanisms that enable the animal to supply the substantial demands of the mammary gland by utilising body reserves of lipid, protein and minerals while maintaining metabolic and physiological equilibrium (Collier 1985).

Nutrient partitioning is controlled by a complex and integrated system involving most if not all tissues in the body (Figure 2.2). There are many control points or rate-limiting steps in numerous metabolic pathways that may be involved in causing variation between animals.

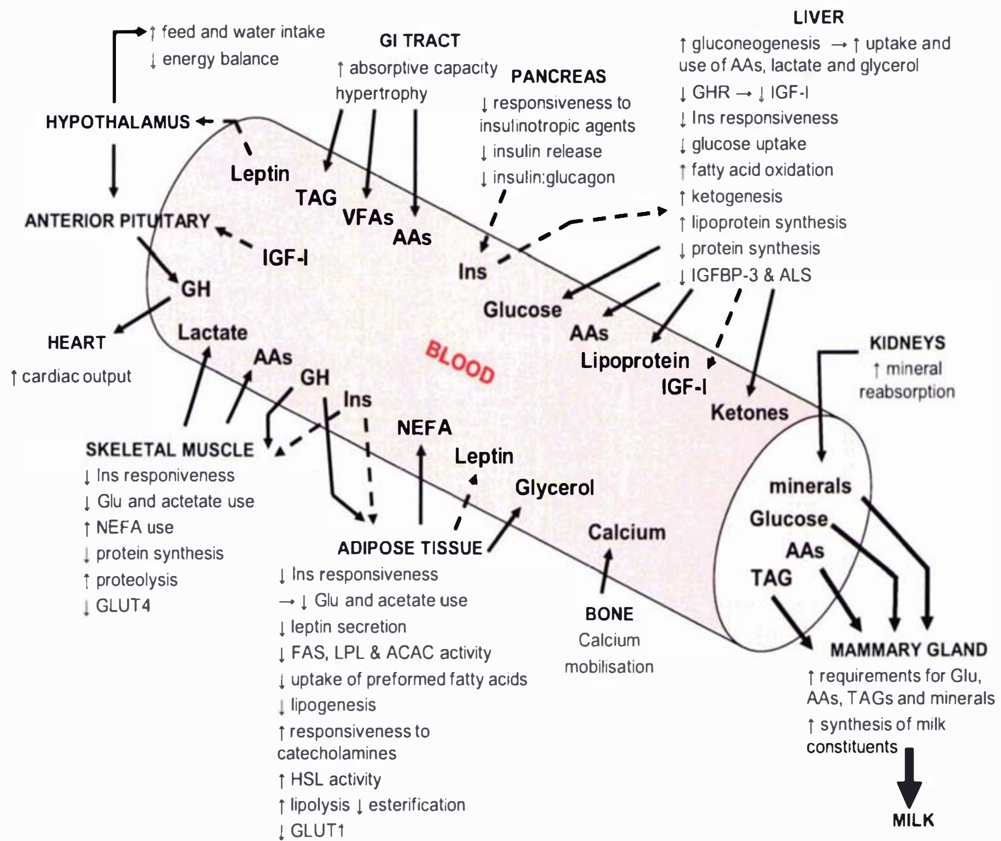


Figure 2.2 Schematic representation of some of the metabolic changes that occur in the periparturient dairy cow. Abbreviations: Ins, insulin; Glu, glucose; NEFA, non-esterified fatty acid; GLUT4/1, solute carrier family 2 (facilitated glucose transporter), member 4/1; FAS, fatty acid synthase; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase; AAs, amino acids; TAG, triacylglycerol; GI tract, gastrointestinal tract; IGF-I, insulin-like growth factor I; GHR, growth hormone receptor; GH, growth hormone; IGFBP-3, Insulin like growth factor binding protein-3; ALS; acid labile subunit; ACAC, acetyl CoA carboxylase; ↑, increase; ↓, decrease; →, which results in. Fluxes: bold line, flux increased; dashed line, flux decreased.

Rate-limiting steps or control points in a pathway or cascade are the most obvious places to target when looking for causative QTL for milk production because even if QTL for the subsequent steps in a pathway were found this will not change the outcome of the pathway unless that the bottleneck created by the rate-limiting step was also improved. An example of a metabolic control points is the decrease in lipoprotein lipase (LPL) activity during early lactation in dairy cows and goats (Chilliard *et al.* 1977, McNamara *et al.* 1987, Shirley *et al.* 1973). LPL is found in the surface of the capillary endothelium of adipose tissue and catalyses the rate-limiting step in the hydrolysis of triglycerides from circulating chylomicrons and very low density lipoproteins (Ren *et al.* 2002) making fatty acids, in the form of triacylglycerols, available for storage in adipose tissue (Hocquette *et al.* 1998). A reduction in the activity of

LPL during early lactation causes a reduction in the uptake and storage of fatty acids by adipose tissue making this energy source available to other tissues to support lactation. The activities of a number of other key enzymes are also altered in adipose tissue with the onset of lactation and will be discussed in a subsequent section of this review.

A second example is the dramatic increase in the number of β-adrenergic receptors per adipocyte that occurs during the periparturient period (Jaster and Wegner 1981). Increased β-adrenergic receptor number coincident with lactation is a mechanism that increases the release of free fatty acids and glycerol from adipose tissue (Jaster and Wegner 1981). Dairy cows differ in the extent to which they mobilise body fat reserves (Bryant and Trigg 1981, Davey *et al.* 1983), it is possible that variation might exist between cows in the control mechanisms involving catecholamines and their receptors on adipose tissue (Bauman *et al.* 1985).

It is important to understand the control of nutrient partitioning during lactation, particularly the rate-limiting points of control. If these rate-limiting points are differentially regulated by genetic variants (alleles) they would be ideal targets when conducting QTL searches. In the following sections the partitioning of nutrients during lactation, summarised in Figure 2.2, will be discussed.

2.5 Carbohydrate Metabolism in the Ruminant

Simple-stomached species digest dietary carbohydrates to glucose and other simple sugars, which are then absorbed into the portal blood to act as a source of glucose for body metabolism. In contrast, ruminants have evolved an alimentary tract that harbour micro-organisms capable of digesting structural carbohydrates, such as cellulose, that constitute plant fibre (Faichney 1993). Most of the dietary carbohydrates undergo microbial fermentation in the reticulo-rumen to produce volatile fatty acids, the most important of which are acetate, propionate and butyrate (Bergman *et al.* 1974, Brockman 1993). As a result, ruminants only absorb small amounts of dietary glucose (Danfær *et al.* 1995), therefore, they must rely almost entirely on

gluconeogenesis in the liver and to a lesser extent in the kidneys to supply their glucose requirements (Bell and Bauman 1997).

Hepatic output of glucose normally accounts for 80-90% of glucose-entry rate and while renal production of glucose accounts for 5-10% of the whole body glucose turnover (Bergman *et al.* 1974) it may increase to 15% during lactation (Brockman 1993). Other adaptations to intermediary metabolism that have occurred in ruminants due to low dietary glucose absorption are limited use of glucose for oxidation and the use of acetate in place of glucose for fatty acid synthesis (Danfær *et al.* 1995). The tissues of ruminants are less responsive to insulin than those of simple-stomached species. It is thought, this is due to the pattern of feeding and the continual fermentation in the rumen, both of which minimise surges in nutrients, particularly glucose, entering the portal vein, decreasing the need for insulin to act rapidly to divert nutrients to storage as glycogen and fat (Vernon and Sasaki 1991). Although little glucose is absorbed from the alimentary tract in ruminants, it still has a central role in their metabolism as in all mammalian species.

2.6 Carbohydrate metabolism in the lactating ruminant

Glucose uptake and utilisation

Lactation greatly increases the glucose requirement of ruminants, for example, basal utilisation rate is approximately 3 times higher in lactating than in dry goats (Debras *et al.* 1989). Moreover, mammary uptake can account for 50-80% of whole body glucose utilisation (Annison 1983, Knight *et al.* 1994) because it is a vital substrate for mammary metabolism and milk synthesis (Bell and Bauman 1997, Collier 1985, Zhao *et al.* 2004). Up to 67% of the total irreversible loss rate of glucose in dairy cows is used for lactose synthesis (Bauman *et al.* 1988).

Lactose, which is unique to milk, is a disaccharide of glucose and galactose (Collier 1985). Because it draws water osmotically into the milk, the rate of lactose synthesis and secretion largely determines milk yield (Danfær *et al.* 1995, Zhao *et al.* 1996). The final step in the

synthesis of lactose is the formation of lactose from free glucose and UDP-galactose. While most of the latter is derived from glucose it may also be derived by gluconeogenesis from glycerol and gluconeogenic amino acids (Collier 1985). However, ruminant mammary tissue does not have the necessary enzymes for synthesising free glucose, so it is absolutely reliant on the uptake of blood glucose for the glucose moiety (Knight *et al.* 1994). Glucose uptake into the mammary gland is considered to be a rate-limiting step for milk production (Zhao *et al.* 2004) thus, a high rate of glucose supply to the mammary gland is a necessity for high milk yields.

Intensive selection for high milk production in dairy breeds has changed metabolism compared to beef breeds (Pareek *et al.* 2007, Shingu *et al.* 2002). Mammary parenchymal tissue mass, the proportion of mammary epithelial cells differentiated to an active secretory state cows and the and the activity per cell found to greater in dairy than in beef cows (Akers *et al.* 2006, Keys *et al.* 1989). In comparative studies, particularly in early lactation, milk production and dry matter intake (DMI) were higher in dairy than in beef cows, a greater proportion of metabolisable energy (ME) intake was partitioned into milk and less into body tissue in dairy cows while body condition score (BCS) and energy and nitrogen balances were higher in beef cows (Pareek *et al.* 2007, Yan *et al.* 2006). Baseline insulin and glucose concentrations and insulin concentrations in response to a glucose challenge were higher in beef that dairy cows (Pareek *et al.* 2007, Shingu *et al.* 2002). When nutrient partitioning was compared in dairy and beef cows fed at two different feeding levels, a higher proportion (66% vs 37%) of the additional ME intake with the high concentrate diet compared with the low concentrate diet was partitioned to milk energy in dairy compared with beef cows, particularly in early lactation (Yan *et al.* 2006). The onset of lactation is associated with a large glucose demand in dairy cows resulting in decreased pancreatic insulin secretion, whereas the higher insulin secretion in beef cows would support greater protein accretion (Pareek *et al.* 2007). Thus, the exceptional demand for glucose experienced during lactation in the high genetic merit dairy cow is not a limitation in lower

producing animals and there have been changes in metabolism relating to the differing selection objectives as a result.

The increase in glucose demand during lactation is met by a number of changes in carbohydrate metabolism (Figure 2.2). The rate of gluconeogenesis increases in the liver with the onset of lactation (Bauman 2000, Vernon *et al.* 1984). Reduced glucose use by peripheral tissues has been indicated by a marked reduction in whole-body glucose oxidation in newly parturient dairy cows (Bennink *et al.* 1972), and is likely to be mediated by very low plasma insulin concentrations and decreased sensitivity of tissues to insulin (Holtenius *et al.* 2003, Lomax *et al.* 1979, Vernon *et al.* 1990). Thus, carbohydrate metabolism and its control play a critical role in milk production.

Circulating insulin concentrations

Despite ruminant tissues being less responsive to insulin than the equivalent tissues in monogastrics, insulin is still the dominant anabolic hormone and regulator of the energy metabolism in resting ruminants (Brockman 1986). Insulin stimulates the uptake and utilisation of glucose and acetate by peripheral tissues, inhibits gluconeogenesis and glucose release from the liver and stimulates lipogenesis (Bassett 1974). In early lactation dairy cows, the concentration of plasma insulin at baseline or in response to insulinotropic agents is much lower than in pregnant or dry cows and remains low for many weeks post partum (Accorsi *et al.* 2005, Gulay *et al.* 2004, Holtenius *et al.* 2003, Komatsu *et al.* 2005, Lomax *et al.* 1979, Lukes *et al.* 1989, Rhoads *et al.* 2004). The fall in insulin concentration during lactation has been attributed to negative energy balance, and loss of pancreatic responsiveness to insulinotropic agents, such as glucose and propionate, and possibly increased insulin clearance rates (Debras *et al.* 1989, Faulkner and Pollock 1990a, Faulkner and Martin 1999, Hart *et al.* 1980, Holtenius *et al.* 2003, Hubinont *et al.* 1986, Lomax *et al.* 1979, Moussavi *et al.* 2007). The calculated secretion rate of insulin was more than twice as high in low- compared with high-yielding cows (Hart *et al.* 1980).

High genetic merit cows have lower ($P<0.05$) plasma insulin concentrations, during lactation, than cows of average genetic merit (Knight *et al.* 2004) and there is a negative relationship between circulating insulin concentrations and milk yield (Walsh *et al.* 1980). Administration of insulin suppresses milk yields in cattle unless exogenous glucose is supplied (Beck and Tucker 1978). The proportion of basal glucose uptake that was mediated by insulin in mid-lactation dairy cows was approximately 7% (Rose *et al.* 1997). This small value was due to the large non-insulin mediated requirement of the mammary gland. When insulin was increased 6-fold above basal concentrations, insulin mediated glucose uptake increased to 38.5% and hepatic glucose production was decreased (Rose *et al.* 1997). Experiments using 4 day hyperinsulinemic-euglycemic clamps in early lactation, in which insulin concentrations were increased 7-fold compared to the pre-clamp period or controls, caused a decrease in plasma NEFA, β-hydroxybutyrate, urea nitrogen, true protein and growth hormone concentrations and increased plasma IGF-I concentrations (Butler *et al.* 2003, Mashek *et al.* 2001). As lactation progresses, milk yield declines and energy balance becomes positive, plasma insulin concentrations gradually increase (Lukes *et al.* 1989, Mashek *et al.* 2001, Shingu *et al.* 2002). Taken together, these experiments illustrate the importance of low insulin concentrations on mediating changes in energy metabolism during lactation, particularly early lactation.

Changes in insulin responsiveness and glucose use in peripheral tissues

The ability of insulin to maximally stimulate glucose utilisation above basal concentrations was greatly impaired in early lactation goats when compared with the dry period (Debras *et al.* 1989). However, when assessed using circulating plasma insulin concentrations, lactating sheep showed a more sensitive ($P<0.01$) response to insulin, with respect to glucose utilisation, than non-lactating sheep (Faulkner and Pollock 1990a). But, overall endogenous glucose production was not suppressed by insulin to the same extent in lactating as in non-lactating sheep, glucose production was 54% of basal production in lactating sheep and 23% of basal in non-lactating sheep (Faulkner and Pollock 1990a). Insulin resistance, both *in vivo* and *in vitro*, was apparent in adipose tissue (Guesnet *et al.* 1991, Vernon and Finley 1988, Vernon and Taylor 1988) and

skeletal muscle (Vernon *et al.* 1990) of lactating ruminants. That is, the sensitivity and responsiveness to insulin were reduced in the peripheral tissues of lactating compared with non-lactating sheep (Vernon *et al.* 1990).

Insulin resistance during lactation does not appear to be the result of a decrease in the number of insulin receptors or impaired binding affinity of insulin receptors in skeletal muscle or adipose tissue (Balage *et al.* 1992, Guesnet *et al.* 1991, Metcalf *et al.* 1986, 1991, Vernon and Taylor 1988, Vernon *et al.* 1981, Wilson *et al.* 1996). Insulin binding was increased in pregnancy and remained elevated during lactation in isolated hepatocytes from cows and sheep (Gill and Hart 1980, 1984). Therefore, the ability of these tissues to bind insulin appears to be unimpaired by lactation, suggesting that adaptations lie downstream of receptor binding (Wilson *et al.* 1996).

Glucose transport across plasma membranes is mediated by members of the facilitative glucose transporter family (solute carriers SLC2A, protein symbol GLUT). GLUTs mediate a bidirectional and energy-independent process of glucose transport in most tissues (Zhao *et al.* 2004). GLUT1 is the most abundantly distributed glucose transporter and mediates basal cellular glucose uptake (Abe *et al.* 2001). The other GLUTs are distributed less widely, GLUT2 to 5 are only present in certain tissues (Zhao *et al.* 1993). GLUT4 is the major insulin-responsive glucose transporter and is most abundant in skeletal muscle (Abe *et al.* 2001, Zhao *et al.* 1993).

Glucose transporters have been reported to change in peripheral tissues in response to lactation (Komatsu *et al.* 2005, Zhao *et al.* 1996). GLUT1 mRNA concentrations in adipose tissue of late or non-lactating cows were six times greater ($P<0.05$) than those in cows at peak-lactation (Komatsu *et al.* 2005). The abundance of GLUT4 mRNA in adipose tissue was not altered by lactation (Komatsu *et al.* 2005). Administration of bovine growth hormone from day 118 to day 181 post-partum decreased ($P < 0.03$) GLUT4 mRNA by 44% in skeletal muscle (Zhao *et al.* 1996). This experiment appears to mimic the increase in the concentration of circulating GH that occurs in dairy cows during early lactation (Bell *et al.* 2000, Rhoads *et al.* 2004). The

results of this study may indicate that one action of increased GH during early lactation is to reduce GLUT4 mRNA, thus reducing glucose utilisation in skeletal muscle. However, another experiment did not find changes in abundance of GLUT4 mRNA in skeletal muscle during lactation (Komatsu *et al.* 2005). Changes in GLUT1 and 4 in adipose tissue and skeletal muscle, respectively, are likely to assist in reducing glucose uptake by these tissues thus making more glucose available to the mammary gland.

Activities of enzymes that utilise glucose are decreased in muscle and adipose tissue during lactation. The activities of the glycolytic enzymes phosphofructokinase and pyruvate kinase decreased in skeletal muscle of lactating compared with non-lactating sheep (Vernon *et al.* 1984, Vernon *et al.* 1987), and could account for the decrease in glucose utilisation in the hind limb during lactation (Pethick and Lindsay 1982, Vernon *et al.* 1990). Pyruvate dehydrogenase activity was also decreased in muscle, due to a fall in the proportion of the enzyme in the active state which is probably the major reason for the increased proportion of glucose-derived carbon released as lactate by the hind-limb during lactation (Vernon *et al.* 1984). Hexokinase and glucose-6-phosphate dehydrogenase activities per milligram of protein increased in the skeletal muscle of lactating compared with non-lactating animals (Vernon *et al.* 1987). Activities of glycerol-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, fatty acid synthetase, 6-phosphogluconate dehydrogenase and acetyl coA carboxylase were decreased in the adipose tissue of lactating ruminants, thus aiding a reduction in glucose utilisation by the adipose tissue (Chilliard *et al.* 1991b, Travers *et al.* 1997, Vernon *et al.* 1991).

Studies in humans have shown that elevated concentrations of non-esterified fatty acids (NEFA) markedly decreased insulin-stimulated glucose uptake in skeletal muscle (Krebs and Roden 2005). Onset of lactation causes a dramatic increase in plasma NEFA concentrations in high producing dairy ruminants (Murondoti *et al.* 2004, Sidhu and Emery 1973). This increase in NEFA concentrations may prevent glucose uptake by skeletal muscle in ruminants as it does in humans.

Changes in the activities of a number of enzymes, in abundance of GLUT1 and 4 mRNA and adaptations downstream of insulin receptors in both skeletal muscle and adipose tissue (Figure 2.2), contribute to the co-ordinated reduction in glucose and acetate use and increasing the availability of nutrient sources for milk synthesis. However, although these changes are important for repartitioning of nutrients during lactation they can not be assessed using metabolic challenges.

The role of growth hormone in insulin resistance

Plasma GH concentration increases with the onset of lactation (Bell *et al.* 2000, Ingvartsen and Andersen 2000, Rhoads *et al.* 2004). Glucose uptake in response to elevated plasma insulin concentrations was inhibited during GH treatment of lactating dairy cows compared with controls (Rose *et al.* 1998, Sechen *et al.* 1990). Treatment with exogenous GH caused a reduction in glucose uptake by the hindlimb muscle in lactating, multiparous dairy cows (McDowell *et al.* 1987). *In vitro* studies have shown that treatment of adipose tissue cultures, taken from lactating sheep, with insulin caused a marked increase in fatty acid synthesis from 24 h of culture onwards but addition of GH to the culture medium inhibited the insulin induced increase in fatty acid synthesis (Borland *et al.* 1994, Vernon 1982, Vernon and Finley 1988). Consequently, in ruminants GH is thought to be antagonistic to the action of insulin, preventing glucose uptake in insulin sensitive tissues, and directing glucose to mammary tissues, which are insensitive to insulin (Rose *et al.* 1996).

The role of glucagon

Glucagon has little effect on the peripheral tissues of ruminants (Brockman and Laarveld 1986, She *et al.* 1999b). The rate of gluconeogenesis is stimulated by glucagon and inhibited by insulin (Danfær *et al.* 1995, Drackley *et al.* 2001). Glucagon stimulated conversion of propionate to glucose in cultured hepatocytes from calves (Donkin and Armentano 1995) and sheep (Faulkner and Pollock 1990b). Glucagon also stimulated glycogen breakdown in cultured hepatocytes from sheep (Faulkner and Pollock 1990b). Studies with multi-catheterised sheep

showed that glucagon stimulated glycogenolysis and gluconeogenesis (Brockman and Bergman 1975, Brockman *et al.* 1975) and infusions of glucagon increased plasma glucose and insulin concentrations in early lactation dairy cows (Bobe *et al.* 2003, Hippen *et al.* 1999). The effects of glucagon appeared to be due to enhanced hepatic uptake of glucose precursors (alanine, glycine, glutamine, arginine, asparagines, serine and lactate) and increased activity of pyruvate carboxylase (PC) (Brockman and Bergman 1975, Brockman and Laarveld 1986). PC is one of the three key enzymes in the gluconeogenesis pathway and plays a vital role in the process of catalysing pyruvate to generate oxaloacetic acid. PC catalyses a thermo-dynamically irreversible reaction, which may be a rate-limiting step in hepatic gluconeogenesis (Xu and Wang 2006). Infusion of glucagon into early lactation dairy cows increased hepatic abundance of PC mRNA (She *et al.* 1999a), which may act to direct carbon, derived from amino acids, to gluconeogenesis (Drackley *et al.* 2001).

Studies have found that plasma glucagon concentration showed a small increase during early lactation in goats and dairy cows and then remained reasonably constant throughout the remainder of lactation (Debras *et al.* 1989, Herbein *et al.* 1985). As mentioned earlier, insulin concentrations are very low during early lactation (Accorsi *et al.* 2005, Gulay *et al.* 2004, Holtenius *et al.* 2003, Rhoads *et al.* 2004) consequently gluconeogenesis is increased in early lactation when the insulin to glucagon ratio is low (Herbein *et al.* 1985). Glucagon concentrations were not altered by infusions of insulin and glucose or glucose alone in dairy cows in early lactation (Laarveld *et al.* 1981) but glucagon concentrations were increased by amino acid infusion in lactating goats (Danfær *et al.* 1995). This supports the suggestion that glucagon stimulates gluconeogenesis to a greater extent from glucogenic amino acids (e.g. alanine, glutamate and glutamine) than from propionate (Brockman and Bergman, 1975) and may be important for stimulating use of amino acids for glucose production in early lactation. Although the circulating concentrations of glucagon may not change greatly during lactation, the low circulating concentration of insulin (Figure 2.2) remove much of the inhibitory action of insulin enabling glucagon stimulation of the rate-limiting steps in gluconeogenesis.

Glucose uptake by mammary gland

Experiments in which hyperinsulinaemia was induced in early lactation dairy cows, showed that insulin did not stimulate mammary glucose extraction and only caused a small increase in amino acid uptake (Laarveld *et al.* 1981, Laarveld *et al.* 1985). Consistent with these studies, the insulin-dependent glucose transporter, GLUT4 mRNA is only present in the mammary gland of lactating cows at low (Zhao *et al.* 1993) or undetectable levels (Komatsu *et al.* 2003, Komatsu *et al.* 2005). GLUT1 was found to be a major glucose transporter in the lactating bovine mammary gland and GLUT1 mRNA level of the mammary gland was three times greater in peak- and late- lactating cows than in non-lactating cows (Komatsu *et al.* 2005, Zhao *et al.* 1993).

From the discussion in the previous sections it can be concluded that increased plasma GH and decreased plasma insulin concentrations, low insulin to glucagon ratio and reduced sensitivity and responsiveness of peripheral tissues to insulin during early lactation result in increased mobilisation of body reserves, increased gluconeogenesis and increased glucose sparing by non-mammary tissues with the overall outcome being greater nutrient availability to the mammary gland. It has been suggested that substrate availability is more important than direct hormonal influences in the regulation of mammary gland metabolism as insulin does not stimulate mammary glucose extraction (Laarveld *et al.* 1981). Thus, the mammary gland seems to be at an advantage over insulin dependent tissues in the competition for nutrients when insulin concentrations are low, such as during early lactation (Laarveld *et al.* 1985).

Differences between high and low genetic merit dairy cattle

It has been demonstrated that plasma insulin concentrations during lactation were lower in cows selected for high milk production than in control cows (Bonczek *et al.* 1988, Hart *et al.* 1980, Knight *et al.* 2004). Other studies found no difference in insulin concentration between selection lines, throughout lactation (Lukes *et al.* 1989). However, Lukes *et al.* (1989) conducted their study on first lactation heifers. Heifers have lower milk production and respond

differently to a glucose challenge than mature cows (Lukes *et al.* 1989) and this may be due to the heifers partitioning some nutrients towards growth. In another study, high BI cows had higher baseline and peak concentrations of plasma insulin than low BI cows, in response to an intravenous glucose infusion, but glucose concentrations did not fall at a greater rate in the high BI cows (Davey *et al.* 1983). Thus, the removal of glucose was less sensitive to insulin concentration in the high BI group (Davey *et al.* 1983), which may indicate peripheral tissues in high BI cows were less sensitive to insulin than in low BI cows. Michel *et al.* (1991) also found that insulin peak in response to a glucose challenge was greater in high BI cows during lactation but, in contrast with Davey *et al.* (1983), found that glucose concentration declined more rapidly in the high BI cows.

Results in calves are also inconsistent. Concentrations of insulin at baseline, and in response to fasting followed by a propionate challenge were not different between high and low genetic merit calves (Land *et al.* 1983, Sinnett-Smith *et al.* 1987). Conversely, baseline plasma insulin and peak insulin in response to a glucose challenge were greater in high BI compared with low BI calves (Mackenzie *et al.* 1988, Xing *et al.* 1993). Many factors influence baseline hormone and metabolite concentrations and responses to a challenge such as energy and protein balance, feed intake (pasture or concentrate), stage of lactation, parity and milk yield (McClary *et al.* 1988, Sechen *et al.* 1990, Waghorn *et al.* 1987, Woolliams and Løvendahl 1991, Xing *et al.* 1991) which may account for the variable results in the aforementioned studies. However, what is clear is that animals that vary in genetic merit for milk production also must vary in their ability to partition nutrients towards milk production, therefore it is important to investigate the ability of animals to control energy homeostasis.

Summary

Metabolic pathways that enable glucose sparing and nutrient mobilisation from body reserves are important during early lactation when there is a massive glucose demand for milk synthesis without a corresponding increase in feed intake (Figure 2.2). It is possible that cows differ in

their ability to spare glucose and mobilise reserves in non-mammary tissues. They may differ in the rate-limiting control points of the metabolic pathways that are involved in these processes. The use of insulin or glucose challenges should give an indication of each animal's ability to partition nutrients, therefore, may also give an indication of their ability to supply nutrients to the mammary gland.

2.7 Lipid Metabolism

The metabolism of energy-yielding compounds in adipose tissues has a pivotal role in supplying the energy demands of lactation (McNamara 1995). High milk yields in dairy cows are related to the ability to mobilise body energy reserves in early lactation (Bauman *et al.* 1985, McNamara and Hillers 1986b). Therefore, identifying genetic markers in hormonal control and metabolic pathways of adipose tissue accretion and mobilisation may be a key area in improving milk production.

In early lactation ruminants, the concentration of plasma NEFAs, glycerol and β-hydroxybutyrate are all higher than in the non-lactating state (Faulkner and Pollock 1990a, Murondoti *et al.* 2004, Sidhu and Emery 1973, Vernon *et al.* 1981). Triacylglycerol uptake from plasma, fatty acid synthesis (McNamara and Hillers 1986c, Vernon and Finley 1988, Vernon *et al.* 1981), mean volume of subcutaneous adipocytes (Vernon *et al.* 1981), and fatty acid re-esterification (McNamara and Hillers 1986c, Metz and van den Bergh 1977, Sidhu and Emery 1973) are all reduced (Chilliard 1999) while lipolytic activity is increased in adipose tissue during early lactation (McNamara and Hillers 1986b, Metz and van den Bergh 1977, Yang and Baldwin 1973) (Figure 2.2). Consequently, there is a decreased uptake of glucose, acetate and preformed fatty acids by adipose tissue (Phillips *et al.* 2003). These changes in adipose tissue metabolism ensure the preferential use of lipogenic precursors by the mammary gland (Vernon *et al.* 2001).

Before parturition, about two thirds of the FFA produced by lipolysis are re-esterified, but during early lactation re-esterification of fatty acids appears to almost cease in adipose tissue of

cattle (Metz and van den Bergh 1977), therefore the ratio of FFA:glycerol release is higher in lactating compared with non-lactating cows (Metz and van den Bergh 1977, Yang and Baldwin 1973). It has been suggested that re-esterification plays a fundamental role in the regulation of lipid mobilisation (modulation of effective release of fatty acids produced by lipolysis). The marked reduction in re-esterification during lactation, and thus in the triacylglycerol-free fatty acid cycle in the adipocyte, permits an economy in the use of adenosine triphosphate (ATP) which normally may represent about 3% of the maintenance requirements (Kelly *et al.* 1991).

Adipose tissue synthesises triacylglycerol primarily using glycerol 3-phosphate produced from glucose and fatty acids, which may be derived from de novo synthesis (mostly from acetate and β-hydroxybutyrate in ruminants) or from hydrolysis of plasma lipoproteins by the action of lipoprotein lipase (Figure 2.3) (McNamara *et al.* 1987, Metz and van den Bergh 1977). Glucose is specifically required for glycerol 3-phosphate synthesis, fatty acid esterification and as part of nicotinamide adenine dinucleotide phosphate (NADPH) needed for fatty acid synthesis (Bell 1995). The rate of acetate incorporation into fatty acids and activities of fatty acid synthetase (FAS), LPL, glucose-6-phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase were lower in early compared with mid-lactation, while malic enzyme did not differ (Chilliard *et al.* 1991b). LPL activity is also decreased in early lactation compared with pregnant or dry cows (Shirley *et al.* 1973). Low lipogenic rates of adipose tissue during early lactation are linked in part to the negative energy balance (Chilliard 1999, Chilliard *et al.* 1991b).

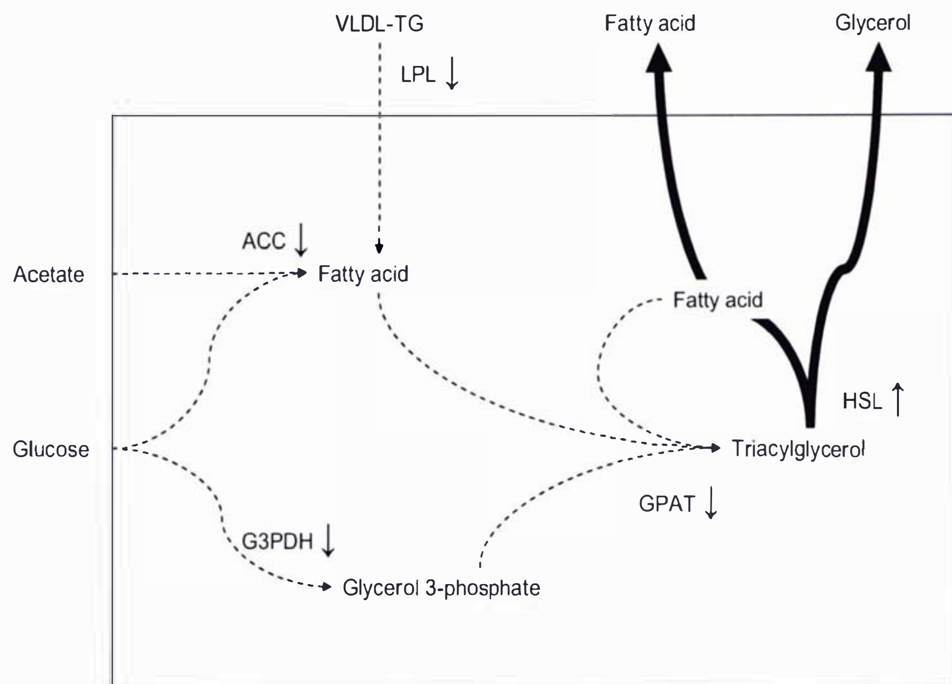


Figure 2.3 Some metabolic pathways of adipocytes and their adaptations to lactation. Enzymes: ACC, acetyl CoA carboxylase; LPL, lipoprotein lipase; G3PDH, glycerol 3-phosphate dehydrogenase; GPAT, glycerol 3-phosphate acyltransferase; HSL, hormone-sensitive lipase. Enzyme acitivty: ↑, increased; ↓, decreased. Metabolic fluxes: bold line, flux increased; dashed line, flux decreased. From: Vernon and Pond 1997

Insulin stimulates lipogenesis and inhibits lipolysis (Bassett 1974). The almost total suppression of adipose lipogenesis and the increased rate of lipolysis, after the onset of lactation, are associated with low levels of plasma insulin and almost complete loss of adipose responsiveness to insulin *in vitro* (Vernon and Finley 1988). These phenomena may be most exaggerated immediately after parturition (Bell 1995). Hyperinsulinaemia, under euglycaemic conditions, in early lactation was shown to cause a decrease in plasma NEFA and glycerol concentrations in cows and sheep, indicating the importance of the very low plasma insulin concentrations on fat mobilisation in early lactation (Andersen *et al.* 2002, Faulkner and Pollock 1990a, Mashek *et al.* 2001). Another experiment using the hyperinsulinaemic-euglycemic clamp technique, in dairy cows 8 days in milk at the start of the study, showed that insulin infusion reduced plasma NEFA concentrations by 86% with a concomitant 27% reduction in

milk fat yield compared with the baseline period (Corl *et al.* 2006). The reduction in milk fat yield was almost exclusively due to a decrease in the use of longer chain fatty acids taken up from the circulation. The changes that occurred with elevated insulin were due to reduced rates of adipose lipolysis, thereby limiting the supply of preformed fatty acid precursors (Corl *et al.* 2006).

Basal release of FFA from *in vitro* adipose tissue cell cultures was inhibited by glucose at concentrations normally found in plasma (3.5 m*M*). Glucose inhibits fat mobilisation by stimulating the re-esterification process through formation of glycerol-3-phosphate (Figure 2.3) (Metz and van den Bergh 1977). However, in adipose tissue samples taken from cows in early lactation, glucose did not have an inhibitory effect on FFA release. Addition of insulin to the medium did not affect the production and release of FFA (Metz and van den Bergh 1977).

Lipogenic capacity of adipose tissue, cultured for 48 h in the absence of any hormones, was markedly decreased (Etherton and Evock 1986, Vernon and Finley 1988). Addition of physiological concentrations of insulin increased lipogenic rate in short-term *in vitro* adipose tissue incubations (Etherton and Evock 1986, Vernon 1982), suggesting that insulin acts to maintain enzyme protein mass in adipose tissue (Etherton and Evock 1986). Addition of GH to adipose tissue cultures containing physiological concentrations of insulin caused decreased ($P<0.05$) rates of fatty acid synthesis from acetate (Vernon 1982).

The dramatically altered response to insulin in early lactation could not be attributed to any change in insulin binding by adipocytes (Vernon *et al.* 1981), implying a post receptor defect or alteration (Wilson *et al.* 1996). Impairment in the insulin signalling system is thought to be downstream of PI3 kinase therefore is at a relatively late step in the insulin signalling system in adipocytes (Wilson *et al.* 1996). It has been suggested that GH may play a role in the altered response of adipose tissue to insulin during early lactation because GH antagonises insulin action. *In vitro* studies in adipocytes have shown that GH prevented insulin activation of actyl-CoA carboxylase (Borland *et al.* 1994, Vernon *et al.* 1991).

Catecholaimes

Catecholamines (adrenaline and noradrenaline) are secreted by the adrenal glands and the sympathetic nervous system. Adipose cells of the dairy cow are sensitive and responsive to catecholamines (Yang and Baldwin 1973). Glycerol and NEFA release from adipose tissue in response to adrenaline was increased from day 30 prepartum to day 30 postpartum and remained elevated until day 240 of lacation (McNamara 1988). Catecholamines can modify adipocyte metabolism to a variable extent, depending upon cell membrane β- and α-2-adrenergic receptors, which stimulate and inhibit lipolysis, respectively. Stimulation of FFA and glycerol release from adipose tissue by noradrenaline, *in vitro*, is increased after parturition (Metz and van den Bergh 1977). From 30 days pre-partum to 30 days post-partum there was a 60% increase in the number of β-adrenergic receptors per cell, although binding kinetics were unchanged (Jaster and Wegner 1981). Because cows differ in the extent to which they mobilise body fat reserves (Bryant and Trigg 1981, Davey *et al.* 1983), it is possible that variation might exist between cows in the control mechanisms involving catecholamines and their receptors on adipose tissue (Bauman *et al.* 1985). Thus, the effect of adrenaline on fat mobilisation may be an important area to investigate for genetic control of milk production.

Treatment of multiparous mid- to late lactation dairy cows with exogenous GH caused greater plasma concentrations of NEFA and glycerol both at baseline and in response to an adrenaline challenge when compared with controls (Houseknecht *et al.* 1995, McCutcheon and Bauman 1986, Sechen *et al.* 1990). At maximal responsiveness to adrenaline challenge plasma NEFA concentrations were increased approximately 6-fold, whereas glycerol concentrations were increased approximately 3-fold. The authors suggested that GH treatment was reducing adrenaline-stimulated rates of re-esterification of fatty acids in adipocytes and increasing lipolysis (Sechen *et al.* 1990).

The mechanism of adrenaline action on lipolysis presumably is due to adenyl cyclase which in turn catalyzes conversion of ATP to adenosine-3',5'-monophosphate, cyclic AMP (cAMP), the

latter then activating hormone sensitive lipase (Yang and Baldwin 1973). The regulation of lipolysis involves cAMP and a signal transduction system that includes stimulatory G proteins (G_s) and inhibitory G proteins (G_i). Catecholamines affect lipolysis through the G_s system, and GH treatment dramatically increases the lipolytic response to catecholamines in lactating cows. The G inhibitory system is a major mechanism by which GH alters lipolysis (Etherton and Bauman 1998).

Leptin

Leptin is a protein hormone synthesised almost exclusively in white adipose tissue. It acts predominantly on regions of the brain involved in regulation or energy metabolism (Leury *et al.* 2003). Leptin is synthesised in proportion to the overall degree of adiposity. Plasma leptin content is a good indicator of body fatness in ruminants fed near their maintenance requirements (Delavaud *et al.* 2000, Rosen and Spiegelman 2006, Trayhurn and Bing 2006). Plasma concentrations of leptin in dairy cows were reduced around the time of parturition and coincided with increased use of endogenous lipids as an energy source but occurred prior to significant depletion of white adipose tissue (Block *et al.* 2001, Holtenius *et al.* 2003, Leury *et al.* 2003). The onset of negative energy balance appears to be partly responsible for the lower concentration of plasma leptin in early lactating dairy cows (Block *et al.* 2001). Increased plasma leptin in response to food intake or increased adiposity is a key signal that decreases food intake and increases energy expenditure by acting on specific receptors in the hypothalamus (Okamoto *et al.* 2001, Rosen and Spiegelman 2006). Thus, the increase in food intake that occurs during early lactation may be influenced by low plasma leptin concentrations.

Summary

Nutrient demands increase dramatically due to the onset of copious milk synthesis and secretion. Early lactation in dairy cows is often associated with a period of negative energy balance as increase in food intake is insufficient to meet the nutrient requirements for milk production and maintenance (de Vries and Veerkamp 2000, Van den Top *et al.* 2005). Fatty

acids are a major source of energy for most tissues during periods of negative energy balance (Vernon 2005). Mobilisation of lipid reserves during early lactation is achieved through a considerable reduction in fatty acid synthesis, a decrease in LPL activity and a decrease in fatty acid esterification. This ensures that precursors for triacylglycerol are available for use in the mammary gland. Thus, the control points in pathways involved in lipid metabolism are important for milk production, particularly in early lactation. Release of NEFA and glycerol from adipose tissue in response to an adrenaline challenge may provide indicators of adipose tissue mobilisation and the ability to supply nutrient to the mammary gland for lactation.

2.8 Protein Metabolism

Much of the ruminants' protein requirements are met by digestion and absorption of rumen microbes in the abomasum and small intestine (Clarenburg 1992). However, the substantial requirement for glucogenic precursors during early lactation can not be met by dietary intake in high producing dairy ruminants (Bell *et al.* 2000). During, at least, the first 7-10 days of lactation, it was estimated that high-yielding dairy cows may need to mobilise up to 1000g of tissue protein/d to satisfy amino acids and glucose demands of the mammary gland (Bell *et al.* 2000). Mobilisation of endogenous protein and increased lactate release from muscle as well as glucose and acetate sparing mechanisms, contribute to supplying some of the glucogenic precursor shortfall in early lactation (Hough *et al.* 1985, Pethick and Lindsay 1982). There is also an increased uptake of ketones, such as β-hydroxybutyrate, for oxidative purposes (Vernon *et al.* 1990).

Insulin stimulates the uptake and incorporation of amino acids into protein and inhibits proteolysis (Bassett 1974). Studies in humans indicate that IGF-I stimulates amino acid uptake and protein synthesis, while insulin inhibits protein degradation in skeletal muscle (Liu *et al.* 2006). Insulin and IGF-I concentrations decrease around the time of parturition and onset of lactation (Abribat *et al.* 1990, Accorsi *et al.* 2005, Block *et al.* 2001, Rhoads *et al.* 2004), which may play a role in increasing precursors available for use in the mammary gland and liver.

There is some evidence for a loss of skeletal muscle protein during lactation. Change in body protein has been calculated in several experiments in high producing dairy cows and have demonstrated a loss (8-21 kg) of body protein in early lactation (Chilliard and Robelin 1983, Komaragiri and Erdman 1997, Phillips *et al.* 2003). Variation in body protein loss between experiments is dependent of the live weight and milk potential of the experimental animals and feeding level (Chilliard *et al.* 1998a). Loss of muscle fibre diameter or area of 9-30% have been reported for the period immediately after calving (Kokkonen *et al.* 2005, Reid *et al.* 1980, Roberts and Turfrey 1983). The proportions of carbon in milk constituents derived from feed and body tissues in dairy cows have been estimated by exploiting the natural variation in the ratio ^{13}C:^{12}C present in C_3- and C_4-plant species (Wilson *et al.* 1988). During early lactation 34% of casein and 24% of lactose in milk was estimated to be derived from body tissue protein in high genetic merit cows (Wilson *et al.* 1988). Interestingly there was considerable variation between animals in the proportional contribution of body-tissue C to individual milk constituents, which was suggested to be associated with genetic merit or the size of the body reserves available for mobilisation (Wilson *et al.* 1988). Another experiment also demonstrated variation between individual cows in protein mobilisation (from 11.2 kg mobilisation to 6 kg retention) during early lactation (Tamminga *et al.* 1997).

Measurement of 3-methyl histidine concentration in plasma or urine on its own or a ratio of 3-MH to creatinine can be used as an indicator of muscle protein degradation (Blum *et al.* 1985, Overton *et al.* 1998, Phillips *et al.* 2003). 3-MH was found to increase dramatically in dairy cows during the first week postpartum, suggesting rapid protein mobilisation during this time. By 2 to 4 weeks postpartum 3-MH and returned to prepartum concentrations, or lower (Blum *et al.* 1985, Kokkonen *et al.* 2005, Overton *et al.* 1998, Phillips *et al.* 2003). However, protein mobilisation may continue beyond the first 4 weeks of lactation. A 30% decrease in plasma creatinine (indicating a decrease in total body protein mass) was found in all treatment groups from day 1 to 21 of lactation. Plasma creatinine continued to decrease in the group that was not

given a glucogenic supplement, until that last sample was taken on day 56 of lactation (Kokkonen *et al.* 2005).

A decreased capacity of skeletal muscle to synthesise protein in lactating sheep is indicated by the decreased ratio of RNA:DNA in early lactation (Smith *et al.* 1981). The absolute synthesis rate of skin protein was 48% lower ($P < 0.05$), muscle protein was 34% lower ($P < 0.05$), and bone marrow was 29% lower (not significant) in lactating compared with non-lactating dairy goats (Baracos *et al.* 1991). The reduction in muscle protein synthesis contributed 72% of the total reduction in protein synthesis in the hind limb tissues of dairy goats during early lactation (Baracos *et al.* 1991).

Amino acids released from skeletal muscle are probably used either for protein synthesis in the mammary gland or gluconeogenesis in the liver. Potential utilisation of amino acids for gluconeogenesis is greatest during the first few weeks of lactation (Overton *et al.* 1998). The upper limit to the amount of body protein that can be mobilised, even if an animal is subjected to severe underfeeding, is about 20% of body protein (Chilliard and Robelin 1983). The contribution of body proteins to the energy requirements of lactation is small but quantitatively important in the ruminant. Body protein mobilisation provides essential amino acids or glucose precursors, which generally constitute the principal limiting nutritional factors for milk production. These nutrients are limiting because of feed intake capacity limitations and energy-nitrogen interactions in the rumen (Chilliard 1999). Experiments in dairy cows have shown that increased dietary protein supply increased milk, protein and lactose yields from week 1 of lactation onwards and induced greater mobilisation of fatty acids from adipose tissue. These findings indicate the limited potential of cows to compensate for a deficit of feed protein supply by mobilising tissue proteins (Kokkonen *et al.* 2002, Ørskov *et al.* 1981), thus support the suggestion that amino acids and glucose precursors are limiting to milk production.

Summary

Mobilisation of body protein reserves may be important in supplying amino acids for protein synthesis in the mammary gland and gluconeogenesis in the liver. However, using a metabolic challenge to assess changes in amino acid uptake and release from body protein reserves would be difficult.

2.9 Liver

The liver has a central role in metabolism. Nutrients absorbed in the portal vein must pass through the liver before reaching the vena cava and ultimately the rest of the body, thus it controls the amount and nature of nutrients available to the rest of the body (Lobley *et al.* 2000, Reynolds 1995). The liver is extremely metabolically active, accounting for 2 to 3% of body mass, but up to 25% of body oxygen consumption and also receives approximately 25% of cardiac output (Lobley *et al.* 2000, Reynolds 1995).

The major metabolic activities of the ruminant liver, with respect to metabolism and milk production, include gluconeogenesis, ketogenesis and synthesis of lipoproteins rich in triacylglycerol for transport to other tissues (Baldwin and Kim 1993). Most of the increased glucose requirement associated with the onset of lactation must be met by increased hepatic gluconeogenesis (Drackley *et al.* 2001) because in ruminants most dietary carbohydrates undergo microbial fermentation in the reticulo-rumen meaning ruminants only absorb small amounts of dietary glucose. Given the importance of glucose to lactation, metabolic processes that occur in the liver are likely to be limiting to production in the lactating cow.

While the metabolic pathways that occur in the liver are crucial in the supply of nutrients during lactation, testing these pathways using a metabolic challenge does not seem feasible.

2.10 Metabolic Pathways Summary

The metabolic control of milk production is an area that has been extensively reviewed and therefore the current work is not an exhaustive or original review of the literature. Rather, emphasis has been placed on metabolic processes that are important in supplying nutrients for lactation and may be quantified using metabolic challenges. Insulin plays an important role in carbohydrate metabolism and alterations in the plasma concentrations and signalling mechanisms during lactation contribute to repartitioning of nutrients towards the mammary gland. GH influences many aspects of energy homeostasis. The increase in circulating GH concentration with the onset of lactation plays an important role in partitioning nutrients towards the mammary gland. GH actions include increasing lipolysis, decreasing lipogenesis, increasing gluconeogenesis, impairing the actions of insulin and increasing cardiac output and blood flow to the mammary gland. Mobilisation of fat reserves during lactation is caused by increased lipolytic activity and decreased triacylglycerol uptake, fatty acid synthesis and fatty acid re-esterification. These changes in adipose tissue metabolism ensure the preferential use of lipogenic precursors by the mammary gland. High milk yields in dairy cows are related to the ability to increase food intake and mobilise body energy reserves in early lactation. Therefore, identifying genetic markers in hormonal control and metabolic pathways of body reserve accretion and mobilisation may be a key area in improving milk production.

2.11 Challenges

There is a well established difference between high- and low-yielding dairy cows in their ability to partition available energy towards milk production (Løvendahl and Sejrsen 1993). Co-ordination of partitioning of energy towards milk production is orchestrated by changes in hormone concentrations as well as tissue sensitivity and responsiveness to these hormones (Bauman and Currie 1980, Bell 1995, Grummer 1995, Vernon 1988, 1998). Early evidence that genetic differences in the tendency to produce milk are associated with the genetic variation in the physiology of energy control in cattle was provided in experiments by Hart *et al.* (1978).

This work has lead to many studies examining the concentrations of metabolites and hormones under a number of different experimental challenge situations with the objective of determining physiological indicators of genetic merit for milk yield in dairy cattle (Woolliams and Løvendahl 1991).

The blood concentration of a hormone represents the balance between the rates of release and degradation of a hormone (Bauman *et al.* 1985, Hart *et al.* 1980). Concentrations of plasma hormones or metabolites measured in blood samples taken as one off samples or serial samples over time have given highly variable and non-repeatable results. These results could not be related to genetic merit of milk production (Hart *et al.* 1981, Osmond *et al.* 1981, Tilakaratne *et al.* 1980, Woolliams *et al.* 1993). Artificial induction of hormone release, through administration of exogenous hormones or metabolites, can efficiently reduce the variation due to normally occurring random spikes and diurnal fluctuations (Løvendahl and Sejrsen 1993).

There are three approaches that have been used to investigate the effects of genetic merit for milk production on physiological indicators (Woolliams and Løvendahl 1991). One approach has been to carry out physiological studies on bulls in progeny testing schemes and correlate results from the physiological tests with the progeny test results (Joakimsen *et al.* 1971, Klindt 1988, Osmond *et al.* 1981, Robinson *et al.* 1992). Another approach was to use animals from selection experiments (Blair *et al.* 1990); groups of animals that, through selective breeding, were divergent in genetic merit for milk production traits (Løvendahl *et al.* 1991a, Mackenzie *et al.* 1988, Min *et al.* 1993, Sejrsen *et al.* 1984, Tilakaratne *et al.* 1980). The other approach was to compare between breeds such as a dairy breed and a dairy beef crossbred animals (Land *et al.* 1983). However, interpretation of physiological results, in young animals, from the third approach is difficult due to possible specialisation of the breeds (Woolliams and Løvendahl 1991).

Differences between cattle in energy and protein metabolism are associated with differences in genetic merit for milk production traits (Mackenzie *et al.* 1988). Challenge methods were used

to try and find reliable physiological indicators of genetic merit for milk yield that can be measured in both sexes before reproductive age (Baker *et al.* 1990, Sejrsen *et al.* 1984, Tilakaratne *et al.* 1980, Woolliams and Smith 1988). Selection of calves rather than adult animals may increase selection accuracy, shorten the generation interval and accelerate the rate of genetic gain (Baker *et al.* 1990, Sasaki *et al.* 2003). The value of an indicator trait in increasing selection response will depend largely on the magnitude of the co-heritability, which is a function of the heritabilites of the indicator trait and of the economic trait to be improved and the genetic correlation between them (Baker *et al.* 1990, Woolliams and Smith 1988). The methods used for assessing potential physiological indicators of milk production will be discussed in the following sections.

Fasting and feeding

Measurement of blood metabolites (such as FFA, urea and glucose) and hormones (such as GH) was the simplest method used in attempts to finding physiological markers for genetic merit for milk production. A number of studies investigated changes in blood metabolites and hormones before and after feeding or during a period of fasting and re-feeding (Høj *et al.* 1993, Mackenzie *et al.* 1988, Min *et al.* 1993, Sejrsen *et al.* 1984, Sinnett-Smith *et al.* 1987, Tilakaratne *et al.* 1980, Xing *et al.* 1991). These studies were conducted in young (3.5 – 10 months of age) animals with high or low genetic merit for milk production. Although the metabolites and hormones did respond to fasting a re-feeding, there were no consistent differences in these responses that could be attributed to genetic merit (Woolliams and Løvendahl 1991). Differences in feed composition (pasture vs. concentrate) and basal plasma concentrations prior to the fast, sex and age affects may have accounted for the differences among the studies conducted (Min *et al.* 1993). Thus, fasting and refeeding methods for assessing physiological indicators have yielded variable results. Using fasting in combination with other challenges may be a better option for generating repeatable and interpretable physiological responses.

Insulin

Changes in energy metabolism are crucial to partitioning nutrients towards the mammary gland during lactation in the dairy cow. Insulin concentrations are very low during early lactation compared with pregnant, dry or late lactation cows (Accorsi *et al.* 2005, Gulay *et al.* 2004, Holtenius *et al.* 2003, Komatsu *et al.* 2005, Lomax *et al.* 1979, Lukes *et al.* 1989, Rhoads *et al.* 2004) and in some cases are lower in cows selected for high milk production than in control cows (Bonczek *et al.* 1988, Hart *et al.* 1980, Knight *et al.* 2004). Pancreatic responsiveness to insulinotropic substances such as propionate is reduced (Holtenius *et al.* 2003, Leury *et al.* 2003, Lomax *et al.* 1979, Sano *et al.* 1993, Shingu *et al.* 2002) and responsiveness of peripheral tissues to insulin is also decreased (Vernon and Finley 1988, Vernon *et al.* 1990) in lactating compared with non-lactating ruminants. Intravenous glucose challenge is a well established method for assessing pancreatic insulin release and the rate of tissue uptake of glucose in ruminants (Åkerlind *et al.* 1999). Therefore, use of an intravenous glucose challenge in heifers, prior to the age that lactation occurs, may provide information on the ability of the animal to produce milk.

Several experiments have shown that insulin concentrations were greater in dairy cattle with a high genetic merit for milk fat or milk yield compared with those that had a low genetic merit (Barnes *et al.* 1985, Mackenzie *et al.* 1988, Xing *et al.* 1993). In other studies, comparison of pancreatic insulin release from high and low genetic merit calves did not show significant differences (Land *et al.* 1983, Sinnett-Smith *et al.* 1987). An experiment in lactating dairy cows showed the removal of glucose was less sensitive to insulin concentration in the high genetic merit group (Davey *et al.* 1983). In support of this suggestion, an experiment in which Coopworth rams were selected on the basis of their response to glucose tolerance tests produced lines of sheep with differences in the action of insulin on peripheral tissues as well as the release of insulin from the pancreas (Francis *et al.* 1990). Conversely, an increased sensitivity to insulin was in high genetic merit 8-month old bull calves (Mackenzie *et al.* 1988) but no difference was found between genetic merit groups in other studies (Barnes *et al.* 1985, Land *et*

al. 1983). An intravenous glucose challenge was conducted on 329 German Holstein progeny test bulls after an overnight fast, they ranged in age from 1 to 5 years (Panicke *et al.* 2002). The calculated correlation coefficients, of the bulls in their 3rd half year of life, showed that glucose half-life and glucose area are suitable parameters for milk yield, fat yield and protein yield (Panicke *et al.* 2002).

Numerous studies have demonstrated differences in energy metabolism among lines of animals divergently selected for aspects of milk production. This indicates that there is a heritable component to energy metabolism. However, the differences between selection lines are highly inconsistent among experiments. This is probably due to differences in many variables such as age, energy balance and feed composition (forage vs concentrate). Nevertheless, as long as environmental conditions are closely controlled, an intravenous glucose challenge is likely to provide useful data on insulin release and sensitivity for phenotype generation.

Growth hormone

GH has a key role in regulation of mammary gland development and lactation, consequently, the GH gene has potential as a marker for genetic variation in milk production (Høj *et al.* 1993). Basal blood concentrations of GH increase during early lactation, and the concentration of GH is greater in dairy cows selected for high milk yield than average or low yielding cows (Beerepoot *et al.* 1991, Hart *et al.* 1978, Kazmer *et al.* 1986, Knight *et al.* 2004, Lukes *et al.* 1989, Roche *et al.* 2006b). In 1937 preprations from the anterior pituitary administered to lactating dairy cows were reported to increase milk yield (Asimov and Krouze 1937). Since that time, treatment of multiparous mid- to late lactation cows with exogenous bovine GH for short periods, 7-12 day periods, has been shown to increase milk production (23-41%) and milk fat percentage compared with controls (Cohick *et al.* 1989, Houseknecht *et al.* 1995, McCutcheon and Bauman 1986, McDowell *et al.* 1987). These experiements along with many others demonstrate the strong galactopoietic effect of GH (McDowell *et al.* 1987). However, not all

experiments have reported a significant relationship between GH concentration and milk production (Herbein *et al.* 1985).

Growth hormone is released episodically from the pituitary into the circulation, resulting in intermittent spikes in blood GH concentration (Anfinson *et al.* 1975, Woolliams *et al.* 1993). Pulse frequency and amplitude vary between and animals and are affected by many factors such as diurnal variations, feed intake and ambient temperature (Evans *et al.* 1991, Løvendahl and Sejrsen 1993, Woolliams *et al.* 1993). Thus, when testing individual animal GH responsiveness, injection of an exogenous GH secretagogue under controlled environmental conditions is the most effective method (Løvendahl and Sejrsen 1993). GH release is highly dependent on many factors including age of calves, energy balance, type and dose of secretagogues and GH concentration prior to secretagogue administration (Høj *et al.* 1993, Løvendahl *et al.* 1991a, Løvendahl *et al.* 1991b, Shingu *et al.* 2001).

Thyrotropin-Releasing Hormone

Thyrotropin-releasing hormone (TRH) is a tripeptide hypothalamic hormone that has been isolated from hypothalamic tissue. Growth hormone releasing hormone (GHRH), TRH and arginine have all been used as GH secretagogues in cattle (Hodate *et al.* 1985, Høj *et al.* 1993, Lapierre *et al.* 1990, Løvendahl *et al.* 1991a, McCutcheon and Bauman 1986, Sørensen *et al.* 2002, Woolliams *et al.* 1993). GH release in response to arginine administration was often small and delayed compared with responses to GHRH or TRH. In some cases there was no GH response to arginine administration (Høj *et al.* 1993, Løvendahl *et al.* 1991a, Romo *et al.* 1997). Thus, arginine will not be considered further in this discussion.

For both GHRH and TRH, size of the GH response may depend in part on the size of the releasable GH pool in the pituitary, and this would be affected by the time since the most recent peak (Løvendahl *et al.* 1991b). GH response to TRH is smaller than the response to GHRH (Løvendahl *et al.* 1991b, Woolliams *et al.* 1993). GHRH has been used successfully in a large number of experiments investigating the secretion of GH in dairy cattle breeds. However,

GHRH is not approved for use in New Zealand, thus could not be used for conducting an endocrine challenge in the current study.

When TRH is administered to cattle, prolactin and GH are released from the anterior pituitary. It is uncertain if TRH controls secretion of prolactin or GH under normal physiological conditions. Both hormones are implicated in several aspects of mammary function (Tucker 1985).

Prolactin has a clearly established role in lactogenesis in the dairy cow (Tucker 2000) and is a primary stimulator of mammary cell differentiation (Akers 2006). Experiments in which the surge in prolactin secretion, which occurs several hours before parturition in the cow, was blocked through the administration of bromoergocryptine caused a marked decrease in milk production and administration of exogenous prolactin prevented the decrease in milk production caused by bromoergocryptine (Akers *et al.* 1981). Prolactin does not appear to affect established lactation in the dairy cow. Increased or decreased plasma prolactin concentrations did not alter milk production in dairy cows either in early or late lactation (Plaut *et al.* 1987).

There were no differences in stimulated secretion or basal prolactin concentrations when cows selected for high yield were compared with controls (Bonczek *et al.* 1988, Kazmer *et al.* 1986). Conversely, basal and response period plasma prolactin concentrations were greater in selection than control cows on the day of parturition but were greater in control cows on days 45 and 90 and did not differ on day 180 post partum (Lukes *et al.* 1989). In another experiment, cows exposed to short day photoperiod during the dry period had reduced prolactin but increased milk yield and expression of prolactin receptor mRNA in lymphocytes and mammary gland compared with cows exposed to long day photoperiod. These authors suggested these findings indicated that greater responsiveness and sensitivity to prolactin during transition to lactation may be associated with an increase in subsequent lactation (Auchtung *et al.* 2005).

TRH acts upon the anterior pituitary to stimulate the release of thyroid-stimulating hormone (TSH) (Veerkamp *et al.* 2003). TSH is recognised as being directly responsible for the release

of thyroxine (T_4) and, in lesser amounts, 3,5,3'-triiodothyronine (T_3) by the thyroid gland (Lacasse *et al.* 1991, Veerkamp *et al.* 2003). T_4 is the predominant thyroid hormone in the circulation but has little biological activity and essentially serves as a prohormone (Akers 2002). Enzymatic 5'deiodination of T_4 within the thyroid and peripheral tissues produces the most metabolically active thyroid hormone, T_3 (Akers 2002, Chopra *et al.* 1978).

Thyroid hormones are involved in metabolic homeostasis and maintenance of lactation (Perera *et al.* 1985). During early lactation in cows there is a decrease in T_4 and T_3 concentrations and a number of studies have found a negative relationship between milk yield and circulating concentrations of thyroid hormones (Hart *et al.* 1978, Nixon *et al.* 1988, Pezzi *et al.* 2003, Walsh *et al.* 1980). Thus, lactation in dairy cows is characterised by a physiological hypothyroid state which is in contrast with the enhancement of milk production induced by T_3 and T_4 administration (Perera *et al.* 1985, Swanson and Miller 1973). Stimulation of milk production through administration of thyroid hormones was found to be unsustainable due to the increase in general metabolism causing severe body weight loss (Blaxter *et al.* 1949, Knight *et al.* 2004). In contrast to the general increase in metabolism that occurs in response to thyroid hormone supplementation, it is likely that organ-specific changes in thyroid hormone metabolism during lactogenesis facilitate adaptation to a lactation state by promoting differential rates of energy utilisation (Akers 2002, Pezzi *et al.* 2003).

A positive genetic association has been reported between daily T_4 degradation and genetic merit for fat-corrected milk yield in AI stud bulls of 3 dairy breeds (Joakimsen *et al.* 1971). There was a tendency for circulating T_4 concentrations during lactation to be lower in dairy cows selected for high milk yield (Bonczek *et al.* 1988, Hart *et al.* 1978). Plasma T_4 concentrations during refeeding were negatively correlated with the breeding value of dairy bulls at 7 months of age (Sejrsen *et al.* 1984). However, other studies did not find differences between selection lines of dairy calves in the plasma concentrations of T_3 and T_4 (Land *et al.* 1983). In a partial least square model the proportion of the variation in energy corrected milk yield explained by blood T_3 concentrations was approximately 19% (range 12-28%), this figure varied depending

on parity, breed and stage of lactation (Ingvartsen and Friggens 2005). Although, there are inconsistent findings with regard to circulating thyroid hormone concentrations in relation to milk production, measurement of TSH may give an indirect indication of genetic potential for milk production.

Adrenaline

Adipose tissue is a vital energy source during lactation in the dairy cow. The plasma NEFA, glycerol and β-hydroxybutyrate concentrations are all higher in early lactation ruminants than in the non-lactating state (Faulkner and Pollock 1990a, Murondoti *et al.* 2004, Sidhu and Emery 1973, Vernon *et al.* 1981). Between 20 to 43% of a cow's lipids may be mobilised during the first two months of lactation to supply the energy demands imposed by milk synthesis (Chilliard *et al.* 1984, Chilliard *et al.* 1991a). During early lactation the NEFA released by adipose tissue contribute directly and substantially to milk fat secretion. It has been estimated that the proportion of milk fat derived from body fat was between 0.54-0.43 in early lactation (Wilson *et al.* 1988). In addition, NEFA mobilisation contributes to oxidative energy metabolism in non-mammary tissues, in doing so, spares glucose and amino acids for use by the mammary gland (Chilliard *et al.* 2000). Therefore, the ability of an animal to mobilise adipose tissue may affect its ability to sustain high levels of milk production, particularly during early lactation when feed intake does not increase as rapidly as the energy requirements.

Catecholamines are among the most effective regulators of adipose tissue lipolysis (Lafontan 1994). Adipose tissues have an increased responsiveness to catecholamines during early lactation (Metz and van den Bergh 1977). This may be due to an increase in the number of β-adrenergic receptors per cell (Jaster and Wegner 1981). Animals with a higher genetic merit for milk production have consistently higher rates of lipolysis and hormone sensitive lipase activity (McNamara 1994). Adipose tissue lipolytic potential can be estimated *in vivo* by plasma glycerol or NEFA response to injection or infusion of catecholamines (Chilliard *et al.* 1998b, Kolver *et al.* 2001, McCutcheon and Bauman 1986, Xing *et al.* 1991). The increase in plasma

NEFA concentrations in response to an adrenaline or fasting challenge were not different between high and low genetic merit selection lines (Mackenzie *et al.* 1988, Michel *et al.* 1991, Xing *et al.* 1991). However, a significant ($P<0.01$) linear relationship was found between daily milk energy secretion and NEFA area response to an adrenaline challenge. The regression accounted for 67% of the variation in milk energy output (McCutcheon and Bauman 1986). NEFA liberated during lipolysis can be released into the circulation or re-esterified into newly formed triacylglycerols. Conversely, glycerol kinase is absent from bovine adipose tissue which means reutilisation of glycerol for triacylglycerol synthesis is negligible (Metz *et al.* 1973) and glycerol released during lipolysis must enter the circulation (Dunshea *et al.* 1990). Plasma NEFA, glycerol and glucose concentrations measured in response to an adrenaline challenge will provide information on the ability of animals to mobilise triacylglycerol from adipose tissue and glycogen from the liver.

Summary

Differences between cattle in energy and protein metabolism are associated with differences in genetic merit for milk production traits (Mackenzie *et al.* 1988). Various challenges have been used in an attempt to find reliable indicators of genetic merit for milk yield. In some experiments differences in hormone or metabolite concentrations were found between animals that differed in genetic merit (Barnes *et al.* 1985, Mackenzie *et al.* 1988, Sejrsen *et al.* 1984, Tilakaratne *et al.* 1980, Woolliams *et al.* 1993, Xing *et al.* 1991, Xing *et al.* 1993). However, the response variables measured were affected by many different factors including age, physiological state and feeding regime, therefore the results across experiments were often inconsistent (Woolliams and Løvendahl 1991). It has been suggested that selection for one physiological indicator is unlikely to result in greater genetic gain than using progeny testing. Rather an index of physiological traits would be necessary to enhance the rate of genetic gain (Blair *et al.* 1990).

There have been very few studies that have reported the selection of animals based physiological traits, such as plasma IGF-I concentration (Blair *et al.* 2002, Davis *et al.* 1995). Those experiments that have been reported show that selection based on a physiological trait does not improve the correlated production traits. For example, after 5 generations of selection for high or low plasma IGF-I concentrations in Romney sheep there was a significant difference in plasma IGF-I concentrations between high, low and control (randomly selected). In contrast there was no difference between lines in lamb weaning weight (Kenyon *et al.* 2007). Thus, attempts to identify physiological indicators of milk production traits that could be used to select high genetic merit animals, of both sexes at an early age, have not been successful. No examples of the application of physiological indicator traits in commercial breeding schemes have been reported.

In the current study, glucose, adrenaline and TRH challenges will be conducted on 18-month-old dairy heifers that are in the first trimester of pregnancy. It is likely that a genotype showing increased milk output will exhibit multiple changes in hormone sensitivity and nutrient flow rather than an alteration in a single pathway. Thus, three challenges were chosen to provide data on a number of metabolic systems regulating nutrient delivery to the mammary gland. This was expected to give a much clearer overall picture of nutrient partitioning.

The challenges were chosen in an attempt to simulate aspects of metabolism that have important roles in supplying nutrients for lactation. Measurement of plasma NEFA, glycerol and glucose concentrations in response to an adrenaline challenge may provide indicators triacylglycerol mobilisation from adipose tissue and glycogen from the liver. Mobilisation of lipids from adipose tissues is likely to affect levels of milk production. Plasma insulin, glucose and NEFA concentrations will be measured in response to a glucose challenge. These variables will be used to quantify pancreatic responsiveness to glucose, uptake of glucose in response to insulin and adipose tissue responsiveness to insulin. Insulin plays an important role in controlling carbohydrate metabolism in the lactating cow (section 2.6). Plasma GH, TSH, prolactin and NEFA will be measured in response to a TRH challenge. Responsiveness of prolactin, GH and

TSH secretion to a TRH challenge may provide useful indicators of milk production potential. In particular, basal or artificially stimulated plasma concentrations of GH are greater in dairy cows and calves selected for high milk yield or yield of a milk component, than average or low yielding cows (Beerepoot *et al.* 1991, Kazmer *et al.* 1986, Løvendahl and Sejrsen 1993, Løvendahl *et al.* 1991a, Lukes *et al.* 1989, Mackenzie *et al.* 1988, Roche *et al.* 2006b). GH is thought to play a crucial role in partitioning nutrients towards the mammary gland during lactation.

Some studies have demonstrated that responsiveness to challenges changes with the age and energy intake of the animals (Løvendahl and Sejrsen 1993, Plouzek and Trenkle 1991). Other studies have shown that endocrine challenges conducted when cows are lactating elicit responses that are more relevant to milk production data compared with challenges conducted in pre-partum or pre-pubertal heifers (Ingvartsen and Friggens 2005, Lacasse *et al.* 1994, Mäntysaari *et al.* 1999, Taylor *et al.* 2006). However, responses to a challenge in the lactating cow may either reflect metabolic pathways that cause differences in milk yield or may reflect differences in energy balance. Thus, responses to metabolic challenges conducted during lactation are confounded with energy balance. Response to metabolic challenges in non-lactating animals may be easier to interpret because energy balance can be controlled more effectively than in the lactating animal. It is anticipated that conducting challenges in 18-month-old heifers that are in the first trimester of pregnancy will minimise complications caused by lactation in interpreting physiological data. There is evidence that the regulation of metabolic homeostasis is programmed early in life (Barker 2004). Therefore, it seems likely that animals that possess desirable metabolic characteristics, in terms of nutrient delivery to the mammary gland during lactation, will give some indication of these characteristics when challenged with hormones or metabolites earlier in life.

2.12 Purpose and Scope

Selection of animals for superior milk production has resulted in changes in the production of, and tissue sensitivity to, key metabolic hormones. The genes regulating these hormones are prime candidates for investigation through the use of metabolic challenges (Roche *et al.* 2006a). Many studies have used metabolic challenges in an attempt to find physiological markers for genetic merit for milk production. Previous studies have not used metabolic challenges to find metabolic QTL.

The work described in this thesis focuses on a number of parameters that describe the essential features of hormone and substrate responses to exogenous hormone or metabolite challenges. It is anticipated that these responses will be indicative of important aspects of metabolic pathways involved in milk production. These parameters will be referred to as "metabolic phenotypes" and were used to search for QTL.

The New Zealand Holstein-Friesian and Jersey breeds are two populations of cows that had been segregated for many generations. As such, they might be expected to differ in their genetic makeup at a substantial number of alleles, some of which might be of economic importance. Therefore the primary reason the New Zealand Holstein-Friesian and Jersey breeds were used in this experiment was that they are likely to differ for a number of milk characteristic traits. In this thesis, QTL were identified by investigating the relationship between genetic (DNA) marker alleles and physical (phenotypic) measurements.

Thus, the current study combined previously used challenge methodology with more recent QTL mapping technology. The objective was to discover QTL that are associated with genes underlying some of the important metabolic pathways involved in milk production. The overall aim of this thesis was to explore the use of metabolic phenotypes as a means of improving the rate of genetic gain in dairy cattle.

CHAPTER 3

Materials and Methods

3.1 Animal Ethics

Ethical approval for all experimental procedures on animals resulting from the endocrine challenges was obtained from the AgResearch Ethics committee, protocol number 532.

3.2 Location

Experiments were conducted at the AgResearch, Tokanui Research Farm on Te Mawhai Road, Kihikihi, Waikato, New Zealand (latitude 6345330 S, longitude 2714480 E, New Zealand map grid reference). 38 04 23 S 175 19 41 E World Geodetic System 1984.

3.3 Generation of experimental animals

The Holstein-Friesian, Jersey crossbred (FJXB) QTL experiment was designed with the primary objective being to identify the chromosomal regions that contribute to the genetic differences between these two breeds (Spelman *et al.* 1998). Reciprocal crosses of purebred or 7/8ths Holstein-Friesian and Jersey animals were carried out to produce six F_1 bulls of high genetic merit. The six F_1 bulls had an average breeding worth of 155 (Spelman *et al.* 2004). The F_1 bulls were sired by 3 Jersey bulls mated to Holstein-Friesian cows and 3 Holstein-Friesian bulls mated to Jersey cows (Spelman *et al.* 2001). These F_1 bulls will be heterozygous at all loci that are fixed (homozygous) for different allelic forms in the two breeds. The F_1 bulls were mated to F_1 cows that were selected from commercial herds. The selection criteria were: a fully recorded three generation pedigree; a level of genetic merit that placed the cow in the top 10% of cows in the country; preferably sired by the same sires as the F_1 bulls (Spelman *et al.* 2001). To generate the 800 F_2 animals, the 6 F_1 sires were contract mated to the selected F_1 cows in approximately 400 commercial herds (Figure 3.1). For logistical reasons contract matings occurred over two consecutive years, 1999 and 2000.

Parentage testing was undertaken with 12 microsatellite markers, sire and dam were tested for F_2 animals, while sire only was tested for the F_1 animals. 157 F_2 animals failed parentage

testing and were eliminated from the trial. The first batch of F_2 animals were born in 2000. The F_2 herd had a breeding worth of 139 (Spelman *et al*. 2004).

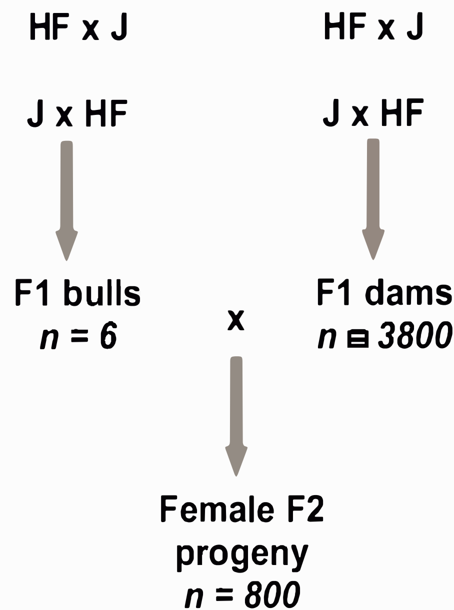


Figure 3.1 Experimental design for Friesian-Jersey crossbred trial. Holstein-Friesian (HF); Jersey (J) From: Spelman *et al.* (2001)

To ensure strain effects were not introduced within the Holstein-Friesian breed, sires and dams of the F_1 animals were selected to minimise the proportion of overseas genes in their three generation pedigree. To avoid inbreeding, cows that were sired by a Jersey were mated to bulls that were sired by a Holstein-Friesian and vice versa (Spelman *et al.* 2001).

The gametes produced by the F_1 animals will carry the alternative forms of all the alleles present in the two parent breeds (Spelman *et al.* 1998) as can be seen in the simple example of segregation in Figure 3.2.

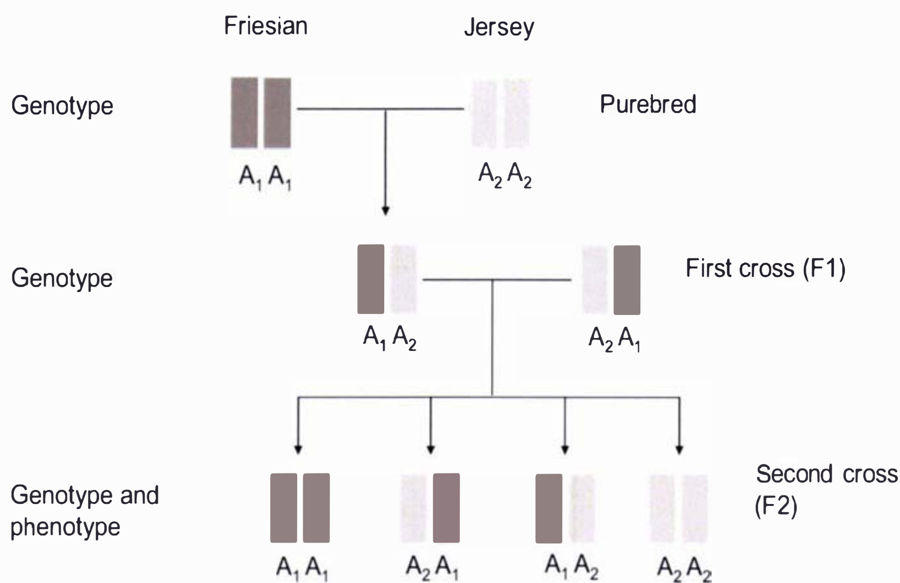


Figure 3.2 Diagrammatic representation of the segregation that occurs in an F_2 experimental design

3.4 Heifer rearing and management

In 2000 (cohort 1) and 2001 (cohort 2) the heifer calves were collected from the farm of their birth when they were 4-10 days of age and delivered to a central rearing facility. Both cohorts were fed milk replacer and a calf starter ration (compound feed). Barley straw was introduced at about 7 days of age to assist in rumen development. Cohort 1 calves were tethered to individual hutches until 10 weeks of age and after that run in mobs of approximately 65 animals. Cohort 2 calves were housed in groups of 20-40, until deemed sufficiently robust to be managed outdoors, usually at 2-3 weeks of age. Calves were weaned between 8-10 weeks of age, when they were eating sufficient pasture and compound feed to maintain satisfactory growth rates. Heifers weighed a minimum of 67.5kg at weaning. After weaning, heifers were grazed on pasture with compound feed supplementation for at least 4 weeks. Silage and compound feed were reintroduced when insufficient pasture was available to meet target growth rates. For ease of management in the rotational grazing system applied on Tokanui Research

Farm, both Cohort 1 and 2 were grazed in six mobs. There were 383 heifers in Cohort 1 and 499 in Cohort 2, therefore, in Cohort 1 there were approximately 65 animals per mob and in Cohort 2 there were approximately 83 animals per mob.

Body weight and condition score measurements were taken fortnightly, height and girth monthly. Monitor animals in each mob were used to assess the health status of each herd e.g. faecal egg counts and mineral status. Puberty was detected by fortnightly measurements of plasma progesterone, plus KAMAR Heatmount Detectors for detection of oestrous behaviour. Bulls were introduced to mobs on 10th October and removed 20th December 2001 for cohort 1. The corresponding dates were 5th October and 15th December 2002 for cohort 2.

Halter training

All heifers underwent a period of halter training in the test facility in preparation for the challenge experiments. Five tie-up sessions per animal occurred in May approximately 6-7 months prior to the challenges occurring. Three further tie-ups per animal occurred in November and December and the animals were rubbed down, especially around the neck area. The purpose of halter training was to ensure the heifers were accustomed to the handling, and the facilities they would be exposed to during the challenge experiment.

Feed

Prior to the challenges, heifers were grazed *ad libitum* on mixed ryegrass/ white clover pasture. Pasture quality may have varied, both within and between years, due to summer weather conditions but no pasture samples were collected to confirm this. Pasture samples were not collected due to an oversight when in the research protocol.

3.5 Experimental animals and groups

Cohort 1 was managed in six mobs (called mob 1 to 6) prior to the endocrine challenges. One week prior to undergoing the challenges each mob was divided into two groups of similar or equal size using ear tag numbers (even and odd numbered tags in different groups). There were 12 challenge groups in Cohort 1 with 31-33 heifers per group (Table 3.1).

Table 3.1 Number of animals per group and cohort, their age (days) and live weight (kg) at the time they were challenged

Group	N	Age, mean ± sd (days)	Age range (days)	Live weight mean ± sd (kg)	Live weight range (kg)
1	32	533 ± 4.3	527-550	355.3 ± 21.5	311-397
2	31	538 ± 5.7	532-558	352.1 ± 25.5	297-420
3	32	535 ± 3.5	528-542	342.6 ± 20.0	305-388
4	31	540 ± 5.0	532-558	344.7 ± 22.5	294-385
5	32	536 ± 4.8	525-545	345.2 ± 24.9	288-414
6	32	543 ± 6.2	530-564	341.6 ± 29.8	301-420
7	32	544 ± 12.1	529-578	343.1 ± 29.5	285-399
8	33	546 ± 8.7	536-568	339.1 ± 28.5	281-379
9	31	544 ± 12.5	528-571	344.4 ± 29.6	291-412
10	32	549 ± 15.1	535-586	347.7 ± 27.5	277-399
11	33	556 ± 19.2	523-582	346.3 ± 25.2	290-399
12	32	561 ± 21.7	528-605	346.3 ± 31.5	263-414
Cohort 1	**383**	**544 ± 14.1**	**523-605**	**345.7 ± 27.0**	**263-420**
13	36	528 ± 11.4	507-557	358.2 ± 21.8	315-404
14	37	528 ± 11.8	508-552	355.6 ± 22.8	317-407
15	37	535 ± 11.3	518-569	358.0 ± 24.9	290-415
16	35	535 ± 13.6	508-559	364.1 ± 21.3	320-421
17	37	530 ± 11.0	516-564	345.7 ± 23.1	303-396
18	35	533 ± 10.1	513-552	344.7 ± 22.4	293-398
19	35	546 ± 11.0	517-564	357.0 ± 22.3	316-408
20	34	550 ± 12.4	524-580	353.8 ± 19.5	305-390
21	33	544 ± 12.0	517-565	346.6 ± 27.7	258-393
22	35	549 ± 13.5	522-574	347.5 ± 24.6	295-417
23	35	552 ± 13.0	521-576	343.8 ± 27.9	275-401
24	37	556 ± 11.7	522-576	340.9 ± 19.0	309-379
25	36	564 ± 12.9	532-586	378.2 ± 33.5	315-451
26	37	563 ± 12.1	532-584	380.3 ± 33.1	270-434
Cohort 2	**499**	**544 ± 17.0**	**507-586**	**355.4 ± 27.7**	**258-451**
Total	**882**	**544 ± 15.8**	**507-605**	**351.1 ± 27.8**	**258-451**

The purpose built, challenge facility could hold a maximum of 37 animals per challenge. The six mobs in Cohort 2 had approximately 83 animals per mob mobs. Thus if they were split into two groups of 41 or 42 animals this would exceed the capacity of the challenge facility.

Therefore, a seventh mob was created in mid-December 2002 by taking between 10 and 15 heifers from each of the six mobs and combining them to create the seventh mob. The seven mobs (called mob 7 to 13) were split in two as described for Cohort 1. In Cohort 2 there were 14 groups with 33-37 heifers per group (Table 3.1).

The 26 challenge groups were balanced for sire, live weight and date of birth. The range in live weights at the time the heifers were challenged was 258-451 kg (Figure 3.3). There was a difference of 98 days between the youngest and the oldest heifer when they were challenged (Figure 3.4 and Table 3.1). The date of conception for each heifer was unknown as a result of using natural mating rather then artificial insemination, therefore, to give an indication of differences between animals in time pregnant when challenged, the number of weeks between being challenged and calving was calculated (Figure 3.5).

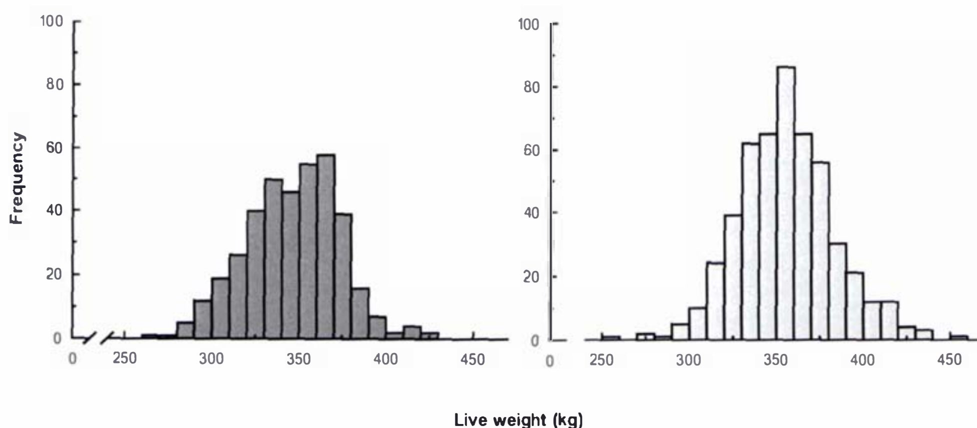


Figure 3.3 Live weight (kg) of heifers at the time they underwent challenges (cohort 1 ■, cohort 2 ■)

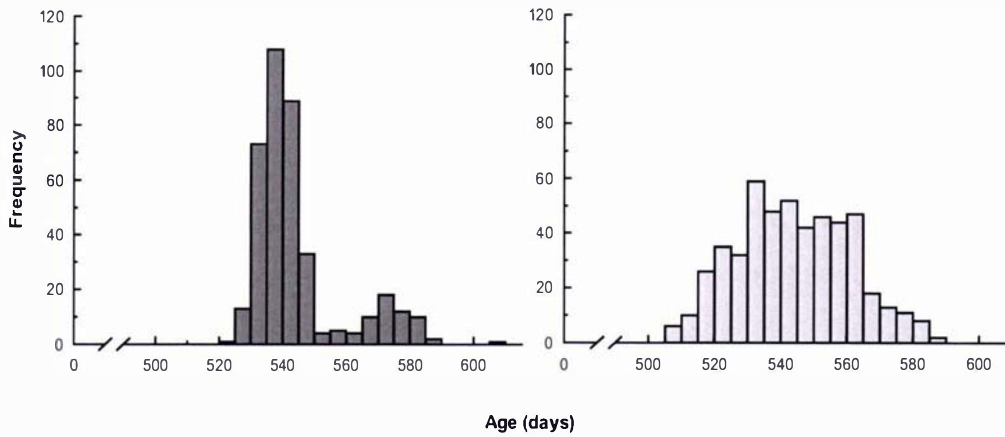


Figure 3.4 Age of heifers (days) at the time they received the challenges (cohort 1 ■, cohort 2 ■)

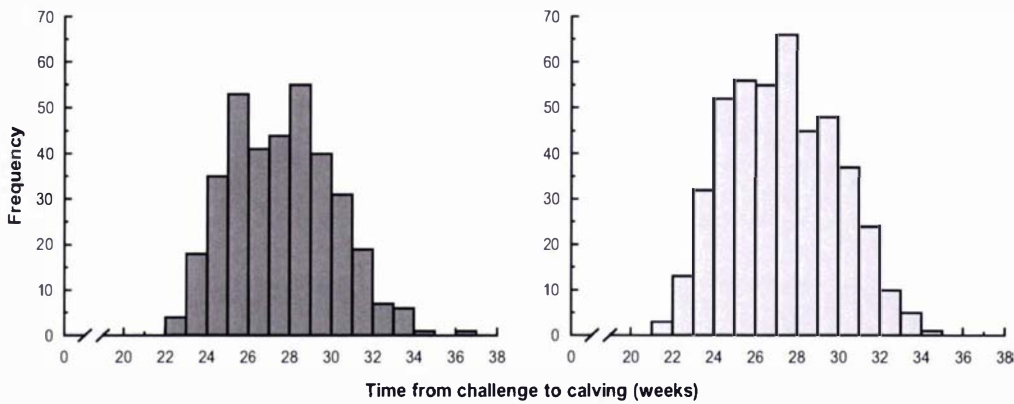


Figure 3.5 Time (weeks) between heifers receiving challenges and when they calved (cohort 1 ■, cohort 2 ■)

Eighty heifers of the 882 challenged (9%) either were not pregnant at the time of challenge or aborted before they reached full term pregnancies. If the heifer aborted far enough through gestation then she was milked, thus she still had lactation data. For analysis purposes these animals were given a calving date of July 15. In 20 of the 26 groups less than 10% of the group did not have a calving date (Figure 3.6). In three groups 20% of the group did not have a calving date, all three of these groups were in cohort 2. The non-pregnant animals remained in the data set for endocrine phenotype QTL analysis but were excluded for the milk production variables.

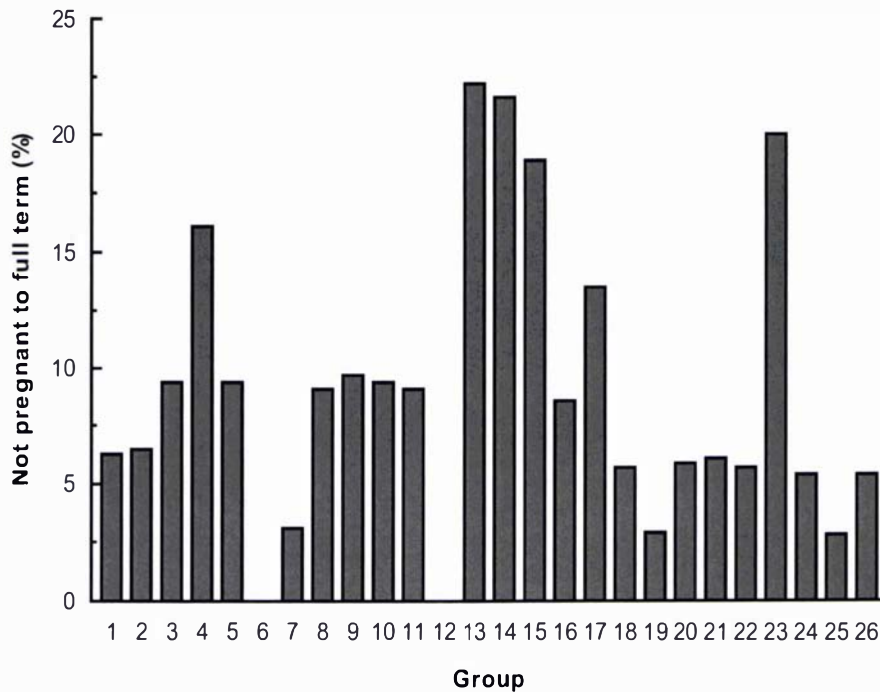


Figure 3.6 Percentage of heifers in each challenge group that did not have a calving date

3.6 Challenge procedure

Time sequence

The experiments occurred in January and February of 2002 (cohort 1) and 2003 (cohort 2). The challenges occurred over a 6 or 7 week period, for cohort 1 and 2 respectively. In 2002 the heifers in group 1 were administered the challenges on 10 and 11 January, two groups were challenged each week subsequent to group 1 with the last group in cohort 1, group 12, receiving the challenges on 19 and 20 February 2002. Group 13, the first challenge group in cohort 2, were challenged on 7 and 8 January 2003 and the last group were challenged on 20 and 21 February 2003.

The sequence of events that occurred for each challenge group (Table 3.2) was as follows. One week before they were to be challenged a mob was yarded and divided into two challenge groups, as described previously. Live weights were measured at this time as described below.

On day 1 of the challenge period the challenge group was brought into the yards and jugular catheters were inserted, described below. The group was returned to pasture for approximately 3 hours before they were brought into a holding yard with a sand base at 19.30h for an overnight fast. When each group was yarded for the overnight fast a health check was carried out to ensure all animals were in good health after the catheters had been in place for several hours, described below.

Two challenges were performed on day two of the challenge period while the animals were kept fasting. The tests were an adrenaline challenge at 8.00h, and a glucose tolerance test at 12.00h. After the completion of the glucose tolerance test heifers were returned to pasture at approximately 15.30h. A thyrotropin-releasing hormone (TRH) challenge was performed on day three of the challenge period. Heifers were returned to pasture after the 4 h blood sample.

Table 3.2 Example schedule for one week during the challenge period. Time sequence not to scale

	Monday	Tuesday	Wednesday	Thursday	Friday
6am		**Group 13** brought into yards	**Group 13** brought into yards	**Group 14** brought into yards	**Group 14** brought into yards
6.15		Halters on and tether in stalls	Halters on and tether in stalls	Halters on and tether in stalls	Halters on and tether in stalls
6.30		Catheter check and flush	Catheter check and flush	Catheter check and flush	Catheter check and flush
6.45		Replace catheters if needed	Replace catheters if needed	Replace catheters if needed	Replace catheters if needed
7.30	Bring **Mob 8** into yards				
8.00	Weigh **Mob 8**, divide into groups **15** and **16**	Commence adrenaline challenge	Commence TRH challenge	Commence adrenaline challenge	Commence TRH challenge
9.00	Finish weighing				
9.15	Download and check weights				
10.00	Return **Mob 8** to paddock – group **15** and **16** kept separate				
11.00		11.10 last heifer finished adrenaline challenge		11.10 last heifer finished adrenaline challenge	
11.15		Rest and water offered		Rest and water offered	
11.45	**Group 13** brought into yards		**Group 14** brought into yards		
12.00	Start inserting catheters	Commence glucose challenge	Start inserting catheters	Commence glucose challenge	
12.15	3 teams: 1 catheter / 15 min		3 teams: 1 catheter / 15 min		
13.00			Last heifer finished TRH challenge, remove catheters		Last heifer finished TRH challenge, remove catheters
13.15			Return **Group 13** to pasture		Return **Group 14** to pasture
14.30		Last heifer finished glucose challenge		Last heifer finished glucose challenge	
15.00		**Group 13** returned to pasture	Return **Group 14** to pasture	**Group 14** returned to pasture	
15.15	Catheterised animals returned to pasture		**Group 13** brought into yards		**Group 14** brought into yards
15.30			Tail bleed **Group 13**		Tail bleed **Group 14**
16.15			Return **Group 13** to pasture		Return **Group 14** to pasture
19.30	**Group 13** brought off pasture into holding area for overnight fast		**Group 14** brought off pasture into holding area for overnight fast		

Live weight measurements

Animals were weighed, one week prior to being challenged, using load-cell scales (Trutest, NZ Ltd). The live weights were used to determine the doses of epinephrine, glucose and TRH given in the challenges.

Catheterisation

Catheter insertion

Jugular catheters (Becton-Dickinson Angiocath™ (product # 382268) – 14 Ga, 3.25"; 2.1 x 83 mm, coated with fluorinated ethylene polymer (FEP) to reduce the incidence of build-up of fibrin clots), were inserted under local anaesthesia (Lignocaine 2%) in a jugular vein on the day before the first challenge. An extension assembly was attached to the end of the catheter allowing easy access to the side of the neck for blood sampling. This catheter was used for both infusion of solution and blood sampling. Once the catheter was in place each animal was given an intravenous infusion, via the catheter, of 10ml of an oxytetracycline based broad spectrum antibiotic (Bivatop® 200, Boehringer Ingelheim (NZ) Ltd) to reduce the risk of infection. The catheters were kept patent overnight with heparinised (50IU/ml) sterile saline. A detailed description of the catheter insertion procedure and the equipment and consumables used can be found in Appendix A. Each group of heifers was taken off pasture at 11:45h, once catheters had been inserted in all animals the group was returned to pasture at approximately 15:00h. The paddock to which they were returned and the holding yards used for the overnight fast had no objects such as tree stumps and the fences, gates and edge of the water troughs had electric fence outriggers attached to them so the heifers were not able to rub and pull their catheter out.

Catheter patency

On both challenge days, heifers were brought into the yards at 6.00h, fitted with halters and taken to their pre-allocated stalls in the challenge facility. Because numerical tag order would reflect the age of the animals, stalls were allocated randomly to avoid any possible confounding effects of age and stall position. The same stall allocation was used on both challenge days. Once the animals were tethered the catheters were checked for patency and flushed with 5ml of

heparinised (50IU/ml) sterile saline. Any animals found to have a non-patent catheter had a new catheter inserted and the event recorded. Thirty-eight, of the 882 (4.3%) heifers that were catheterised for this trial, needed a second catheter to be inserted, either because the catheter was no longer patent or it had been pulled out through the animal rubbing.

Catheter removal

Following the collection of the 4 h blood samples, for the TRH challenge, heifers were injected intravenously, through the catheter, with 10 ml of Bivatop 200 (Boehringer Ingelheim (NZ) Ltd) to reduce the risk of infection, then the catheters were removed. Pressure was applied to the site for a minimum of 1 minute to ensure the puncture wound into the vein closed adequately. The heifers were then returned to pasture.

Health management

The animals were monitored closely for ill-effects on their health caused by the experimental procedure, in particular in relation to the indwelling catheters. Whilst catheters were in place, and for 5 days after catheter removal, the animals were checked twice daily. They were then checked once a day for a further 5 days. If any problems were noted the on-call veterinarian was to be notified and action taken. No animals required veterinary attention in relation to the procedures conducted in this experiment.

Challenges

Adrenaline challenge

The product used for the adrenaline challenge was (±)-Adrenaline hydrochloride (Sigma Aldrich, product number E4642, batch number 110K1576). The dose administered for the adrenaline challenge was 1.4 μg per kilogram of heifer live weight, this dose was used successfully, in an experiment by Kolver *et al.* (2001), to elicit an increase in NEFAs, glycerol and glucose above baseline concentrations in early lactation Holstein-Friesian cows. The concentration of the stock solution was 80 μg per ml of sterile saline. Adrenaline HCl solution is light and air-sensitive therefore, the stock solution was not prepared until the night before use

to minimise any loss of activity. Syringes filled with adrenaline solution were wrapped in aluminium foil to protect the contents from light before administration. L-ascorbic acid was added to the stock solution to act as an antioxidant. Methods used for mixing up challenge stock solution and calculating the dose for each heifer are given in Appendix B.

The adrenaline challenge was administered through the catheter as a bolus dose over a 10 second period. Blood was sampled serially through the catheter at -20, -10, -5, 4, 10, 15, 20, 30, 45, 60, 90, 120 minutes relative to the time of adrenaline injection. The catheter was flushed with 5 ml heparinised (50IU/ml) sterile saline between bleeds. Blood was transferred to 10 ml heparinised blood collection tubes (Becton Dickinson Vacutainer®, Becton-Dickinson and Company, USA) and stored on ice.

Glucose challenge

For the glucose challenge, a dose of 0.3 g glucose (Bomac Laboratories Limited, Wiri Station Road, Manukau City, Auckland, New Zealand) per kilogram of heifer live weight was administered as a bolus injection of the stock solution (400 g/L) from 500 ml Bomaflex ready to use packs (separate pack for each animal). Refer to Appendix B for a description of the methods used for calculating the dose for each heifer.

The glucose solution was administered through the catheter over a period of 1-2 minutes. The exact time taken for each animal to receive her dose was recorded. Blood was sampled serially through the catheter at -20, -10, -5, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120 minutes relative to the start of the glucose administration for the pre-challenge samples and relative to the end of glucose administration for the samples collected after the challenge. The catheter was flushed with 5 ml heparinised (50IU/ml) sterile saline after each sample collection. Blood samples were transferred to 10 ml heparinised blood collection tubes (BD Vacutainer®, Beckton, Dickson and Company, USA) and stored on ice.

After the completion of the glucose tolerance test all heifers received a 10 ml dose of Bivatop 200 (Boehringer Ingelheim (NZ) Ltd) through the catheter followed by a 10 ml flush with heparinised saline. Animals were returned to pasture at approximately 15.30h.

Thyrotropin-Releasing Hormone challenge

The product used for the TRH challenge was Sigma Aldrich, product number 83177, Thyrotropin releasing hormone BioChemika ≥98.0% (HPLC), batch number 361393. The dose administered was 0.3 μg TRH per kilogram of heifer live weight. The concentration of the stock solution was 15 μg per ml of sterile saline. The TRH solution is light and air sensitive, therefore, the stock solution was not prepared until the night before use to minimise loss of activity. Syringes filled with TRH solution were wrapped in aluminium foil to protect the contents from light before administration. The method used for mixing challenge stock solution and calculating the dose for each heifer is in Appendix B.

The TRH challenge was administered through the catheter as a bolus over a 10 second period. Blood samples were collected at -20, -10, -5, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150 minutes relative to the time of the TRH infusion. The catheter was flushed with 5 ml heparinised (50IU/ml) sterile saline after each sample was collected. Blood from samples collected from -30 to 150 minutes were transferred to 10 ml heparinised blood collection tubes (BD Vacutainer®, Beckton, Dickson and Company, USA) and stored on ice and processed as described in the next section.

Blood processing

All blood samples collected were processed in the same way. Plasma was separated by centrifugation (2700rpm, 5°C, 5min) within 2 hours of collection. The plasma was dispensed into 6 pre-labelled cryovials, refrigerated at 4°C then at the end of the each challenge day transported on ice to the storage facility where they were placed in -86°C freezers until analysed for hormones and metabolites.

3.7 Hormone Assays

All the hormone and metabolite assays were carried out in a commercial laboratory, Alpha Scientific Ltd. (Hamilton, New Zealand) unless otherwise stated.

Growth Hormone

Concentrations of GH in plasma were determined using double antibody radioimmunoassay that was validated for bovine use by Alpha Scientific Ltd. GH (bovine GH, AFP11182B) for iodination and the standards, and also the primary antibody of rabbit, anti-Ovine GH-3 (AFP AFP0802210Rb) were obtained from the National Hormone and Peptide Program, California, USA courtesy of Dr Parlow. The GH was iodinated using the Iodogen method (Appendix C). The second antibody (sheep anti-rabbit) IgG was raised in-house.

Briefly, on day 1 standards and controls (100µl standard or control, 100µl filler plasma and 100µl protein buffer), samples (100µl sample and 200µl protein buffer) had primary antibody (100µl, at a 1:130,000 dilution, 10,000 to 15,000 counts per minute) added and were incubated for 24 hours at room temperature. Iodinated GH was added the next day (100µl) and incubated for a further 24 hours at room temperature. The second antibody was added on day 3 (100µl, 1:80,000 dilution) and tubes were incubated for ~18 hours at 4°C. Finally, 1ml of wash buffer was added, tubes were centrifuged for 20 minutes at 2500rpm and at 4°C; the supernatant tipped off and counts per minute determined on a gamma counter (Packard Cobra-II, Auto Gamma).

The intra-assay coefficient of variation (CV) was 9.67% and the inter-assay CV was 19.01%. The maximum detectable concentration for GH assay was 40 ng/mL. The minimum detection limit for the GH was 0.25 ng/mL.

Insulin-like growth factor I

The concentration of IGF-I was measured using a two-site immunoenzymometric assay (OCTEIA IGF-1; Immunodiagnostic Systems Ltd, Boldon, U.K.) based on the work of Daughaday *et al.* (1980). The mean value of control 1 was 56 µg/l with a CV of 8.4% and the mean value of control 2 was 208 µg/l with a CV of 8.2%.

Prolactin

Concentrations of plasma prolactin were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bovine Prolactin, EIA-1549, DRG International Inc., Mountainside, NJ, USA) based on the sandwich principle. Three controls were run, a high, medium and low concentration control. The mean and inter assay CVs were: high 48.9 ng/ml and 11.3%; medium 22.7 ng/ml and 13.9%; low 8.3 ng/ml and 30%.

Thyroid Stimulating Hormone

Concentrations of TSH in plasma were determined using double antibody radioimmunoassay as was validated for bovine use by Alpha Scientific Ltd. TSH (bovine TSH, AFP8755B) for iodination and the standards, and also the primary antibody of rabbit, anti-Bovine TSH (AFP AFP642482) were obtained from the National Hormone and Peptide Program, California, USA courtesy of Dr Parlow. The TSH was iodinated using the Chloramine T method (Appendix C). The second antibody (sheep anti-rabbit) IgG was raised in-house.

Briefly, on day 1 standards and controls (100µl standard or control, 100µl filler plasma and 100µl protein buffer), samples (100µl sample and 200µl protein buffer) had primary antibody (100µl, at a 1:70,000 dilution) added and were incubated for 24 hours at room temperature. Iodinated TSH was added the next day (100µl) and incubated for a further 24 hours at room temperature. On the third day, the second antibody was added (100µl, 1:75,000 dilution) and tubes were incubated for ~18 hours at 4°C. Finally, 1ml of wash buffer was added, the centrifuge was primed to 4°C before use, and tubes were centrifuged for 20 minutes at 2500rpm; the supernatant was decanted and counts per minute determined on a 1470 Wizard Automatic Gamma Counter (Perkin-Elmer Life Sciences, Wallac, Turku, Finland).

Insulin

Insulin concentrations in plasma were measured in a chemiluminescence assay as per the method given in the IMMULITE® Insulin (PILKIN-6, 2002-07-18) kit insert in the Alpha-Scientific Ltd Laboratory, Hamilton, New Zealand. The assay uses monoclonal and polyclonal

antibodies for the capture and detection, respectively, of insulin and was carried out in the Immulite® (Diagnostic Products Corporation, Los Angeles, CA, USA). The mean, standard deviation and inter assay CVs were: high 3.62 ± 0.19 ng/ml and 5.23%; low 1.45 ± 0.08 ng/ml and 5.18%.

3.8 Metabolite Assays

Metabolite concentrations were determined by Alpha Scientific Ltd, Hamilton, New Zealand using enzyme immunoassay, read on a spectrophotometric auto-analyser (Hitachi 717, Hitachi Ltd, Tokyo, Japan). Intra-assay coefficients of variation were not assessed by Alpha Scientific Ltd for the metabolite assays.

Non-esterified Fatty Acids

Concentrations of NEFA in plasma samples were determined using a commercially available acyl-CoA oxidase-based colorimetric kit (Wako NEFA-C, Wako Pure Chemical Industries, Osaka, Japan). The inter-assay coefficient of variation was 5%.

Glucose

Plasma glucose concentrations were determined using the Roche Glucose HK liquid assay kit (catalogue number 1447521, Roche Diagnostics Corporation, Indianapolis, IN, USA). This kit is based on the work of Peterson and Young (1968). The inter-assay coefficient of variation was 3%. The minimum detectable concentration of this assay is 0.11 mmol/litre.

Glycerol

Glycerol concentrations in plasma samples were determined using an enzymatic, colorimetric method using a commercial kit (Randox glycerol kit, catalogue number GY 105, Randox Laboratories Ltd, Crumlin, UK) or (Randox, Mississauga, ON, Canada). The inter-assay coefficient of variation was 5%. Minimum detectable concentration for glycerol was 1 mmol/l, therefore anything less than 1 mmol/l was recorded as zero.

3.9 Other data collection

Milk Yields

A very dry summer, resulting in insufficient food supply to support optimal lactation, occurred during the first lactation of cohort 1. To reduce the impact of feed and weather differences between the cohorts, phenotypes generated from the second lactation data were used for conducting QTL searches in this study.

Daily milk volumes, both am and pm, were collected. In addition, every fortnight milk fat, milk protein, casein, and lactose yield over a 24 hour period were collected (Spelman *et al.* 2004). Once the animals had calved they had weekly condition scores and live weight measurements at least twice a week. During the second lactation for cohort 1, and the first lactation for cohort 2, an automatic weighing system was installed in the milking shed which ensured that on average over 80% of the animals had two live weights recorded each day (Spelman *et al.* 2004). There was not a 100% rate or recording animal weights twice a day due to errors that occurred in the weighing process or due to other unknown factors, for example, if a cow was not correctly on the weighing platform an over or under estimation or the weight would have occurred and the measurement was not entered into the database.

Genotyping

Genomic DNA was prepared from whole blood from a total of 1679 animals within the trial pedigree (846 F_2 daughters, six F_1 sires, 796 F_1 dams, and 13 selected F_0 sires). An initial whole genome scan was conducted by genotyping each animal for 283 microsatellite markers, obtained primarily from published marker maps. The mean interval between microsatellite markers was 10.56 ± 7.67 cM. This marker density was considered adequate for an initial investigation of the genome. This map will be added to with a 50,000 SNP genome scan on which the phenotypes will be run again and will provide greater accuracy of estimation of QTL locations due to the greater density of markers.

3.10 Data Processing

Approximately 27,300 blood samples were collected from the 882 heifers that were challenged, as a result, 164,000 cryovials of plasma were stored and subsequently analysed for 1-5 metabolites/hormones. Despite application of quality control procedures to each stage of sample collection, storage, analysis and data entry, errors were still introduced. Because of the large scale of this trial, it was not possible for one person to have control over every process, meaning that there was a very real opportunity for errors to occur. Therefore, to have confidence in the data systematic checks were conducted.

Data cleaning is the process of detecting, diagnosing and editing faulty data (Van den Broeck *et al.* 2005) and was carried out on the data set used for this thesis to correct errors or at least minimise their impact on the study. Random errors in a large data set such as in the current study are expected but would not have a major influence on the results, rather would simply contribute to the error variance. Conversely, systemic errors may have a considerable impact on the outcomes. A number of systemic errors were identified in the endocrine challenge data (Table 3.3). These systemic errors generally affected the results of all the animals in a challenge group for a particular time point, causing the effect of challenge group on the endocrine results to be greater than it should be. Therefore it was important to identify and correct these types of errors to ensure the most accurate data set was used to commence analyses.

Response curves of each animal and the means per challenge group were plotted and assessed visually. General trends and patterns of responses were identified and data points that did not follow these general trends or apparent systemic errors (Figure 3.7 to 3.9) were noted for further investigation. Once identified, analysis of the questionable data were undertaken to determine whether it was valid or erroneous. The major types of errors found are given in Table 3.3.

Table 3.3 Description of the types of errors identified in the data, how these errors compared with the second database created from the original emails sent from Alpha-Scientific containing the results and the action taken due to discrepancies found

Original database error	Second database	Outcome/action
Missing data points	Some of these were found to exist in the emails database	Where there was a result entered in the emails database this replaced the missing data point in the LIC database
Zero values in the middle of a response curve	In some cases the comment for the data point said variable, insufficient, aliquot missing or no tube	When one of these comments was present the zero value in the LIC database was changed to missing
Two consecutive data points with exactly the same value (example in Figure 3.8)	One of the two values was generally found to be different in this database	When differences between the two databases existed the emails result was entered into the LIC database
Data points 10-fold greater or less than the rest of the results (example in Figure 3.7)	Often this problem was found to be the wrong set of data being entered, for example for group 10 the 5 minute NEFA results had been entered in as insulin results and visa versa	Data was corrected in the LIC database once confirmation of correct results had been made in the emails database
Group 2, 2 minute insulin results were the similar to baseline (Figure 3.6)	The same results were in this database but it was clear from plotting the data these points were incorrect	Plasma samples re-analysed, new results entered into the LIC database
Individual data points that were highly unusual compared with the rest of the response curve	Data point checked in emails database	If different, the result was changed in the LIC database

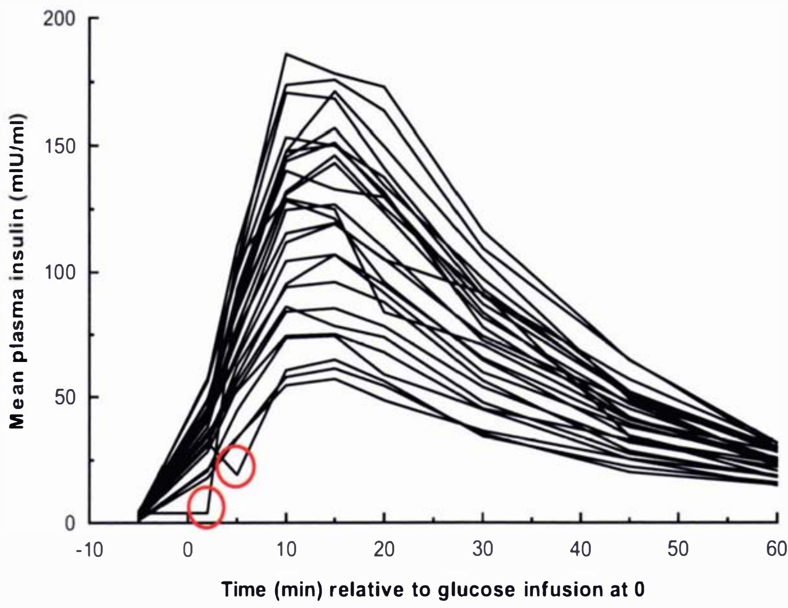


Figure 3.7 Mean insulin release for each challenge group before and after a glucose infusion at time zero. Red circles show examples of errors in the data.

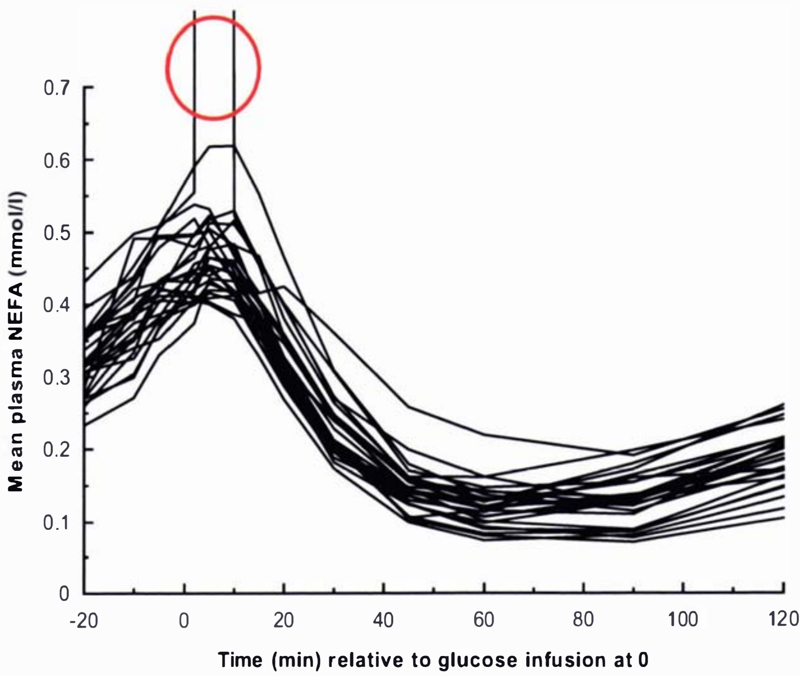


Figure 3.8 Mean NEFA release for each challenge group before and after a glucose infusion at time zero. Red circle highlights an error in the data.

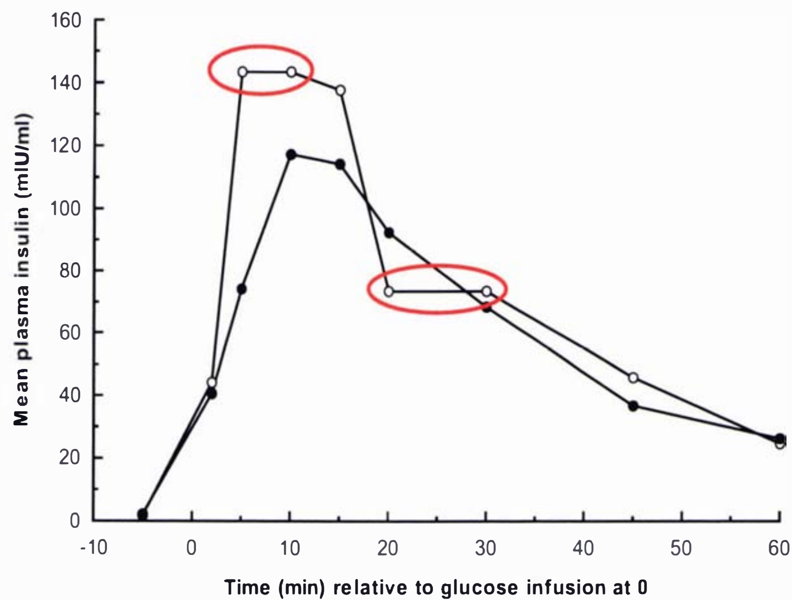


Figure 3.9 Mean insulin release for two subgroups in group 4 before and after a glucose infusion at time zero. Red ellipses highlight consecutive time points that had the same mean value for one subgroup.

Data generated from laboratory analyses conducted by Alpha-Scientific Limited were sent as small subsets in emails to LIC. Emails that had been archived were obtained from LIC and used to generate a new database of results. The questionable data identified in the LIC database were compared with the results in the email database. Where differences were found these were assessed and corrections made to the LIC database.

A total of 87,736 data points were plotted and viewed, 1,415 (1.6%) were identified as questionable data and investigated further. Following diagnosis, 803 (0.9%) data points were found to be incorrect and the value changed. Reports of data error rates in the literature range from 0.34 to 15% over a wide range or study types including clinical laboratory and professional data entry quality control studies (Kawado *et al.* 2003, Nibler 1995, Plebani and Carraro 1997). Studies have indicated that the proportion of the mistakes in laboratory testing can be distributed over 3 areas: pre-analytical 40-68%, analytical 4-13% and post-analytical 18-55% (Boone *et al.* 1995, Plebani and Carraro 1997). Data cleaning in the current study could only assess analytical and post-analytical errors, it seems likely that errors introduced in the pre-analytical stage also exist, however, it was not feasible to assess these errors. Therefore, it is

probable that errors still exist in the current study data set. The majority of errors identified during the data cleaning process were due to incorrect values being entered into the database. For future work of this nature it is important that the person entering metabolic data has a strong understanding of the expected concentrations for each type of analyses so that they can recognise when values that are well outside the expected range are being entered and the error corrected immediately. In addition, double entry of the data is recommended as a means of reducing typographical errors (Kawado *et al.* 2003).

3.11 Data adjustment

Details of the data analyses and the resulting models that were used to adjust the data are discussed in this section.

Unbalanced maternal grandsire distribution

The distribution of maternal grandsires across the F^2 population was unbalanced. In the data set there were 33 maternal grandsires that had five or less F^2 heifers and 30 maternal grandsires that had six or more F^2 heifers. The unbalanced distribution of the maternal grandsires across the F^2 population caused the maternal grandsires to have a disproportionately large influence on the residuals when included in the model. Several ways of grouping the maternal grandsires with only a small number of offspring was investigated. All methods of grouping were done within breed, i.e., Holstein Friesian and Jersey. The F^2 animals were grouped when there were 3 or less, 5 or less or 9 or less F^2 animals per maternal grand sire. The year of the maternal grandsires' birth was also used to group within the aforementioned groupings, this was to ensure that genetic improvement in the maternal grandsires over time was taken into consideration.

The variation explained by the model was not increased by dividing the groups up by year of birth of the maternal grandsires. Grouping F^2 heifers with maternal grandsires that had 9 or less F^2 animals did not explain anymore of the variation than when the criterion was 5 or less F^2 heifers. Therefore, animals were grouped within breed when the maternal grandsire had 5 or less F^2 animals in the trial.

Data analysis

All fixed effects and co-variates were tested at each time point, using PROC GLM in SAS (version 8.2, 1999-2001 by SAS Institute Inc., Cary, NC, USA), to establish which ones have a significant influence on the plasma concentrations of hormones and metabolites tested. Environmental effects were confounded within challenge group. Therefore, environmental variables were removed from the model and group nested within year was included to account for all affects which occurred at a group level. Possible interactions were also tested but none were included because they were not significant at more than 3 sample times. The models used for the data from each challenge are shown below. Residuals were obtained using these models and added to the overall group mean, to obtain adjusted results. These adjusted results were used for analyses and generating phenotypes.

Models

Glucose challenge: $y_{ijklmnop} = \mu + y_i + g_j(y_i) + s_k + m_l + age_m + wgt_n + lwg_o + e_{ijklmnop}$

Adrenaline challenge: $y_{ijklmnop} = \mu + y_i + g_j(y_i) + s_k + m_l + age_m + wgt_n + lwg_o + stall_p + e_{ijklmnopq}$

TRH challenge: $y_{ijklmnop} = \mu + y_i + g_j(y_i) + s_k + m_l + age_m + wgt_n + wtc_o + lwg_p + stall_q + e_{ijklmnopqr}$

where: μ is the overall intercept; y_i is the fixed effect of the year in which the heifer underwent the endocrine challenges; $g_j(y_i)$ is the fixed effect of challenge group nested within year; s_k is the fixed effect of sire (each heifer was the offspring of one of six sires); m_l is the fixed effect of maternal grandsire (maternal grandsire was grouped within breed if there were 5 or less F^2 heifers) ; age_m is number of weeks of age at the time of challenge fitted in the model as a covariate; wgt_n is the live weight at the time of challenge fitted as a covariate; wtc_o is the number of weeks between being challenged and parturition fitted as a covariate; $stall_p$ and $stall_q$ are the fixed effect of stall (within group allocation individual tie stalls, grouped in sets of two (TRH challenge) or three (adrenaline challenge) (adjacent animals)); lwg_o and lwg_p are the daily live weight gain calculated from the difference between challenge weight and a weight

measured at the end of each cohorts challenge period fitted as a covariate; $e_{ijklmnop}$ and $e_{ijklmnop}$ and $e_{ijklmnop}$ are the random residual.

CHAPTER 4

Description of response variables and phenotypes

4.1 Introduction

The objectives for this chapter were to choose a number of parameters that described the essential features of hormone and substrate responses to exogenous hormone challenges. These parameters will be referred to as "metabolic phenotypes" and will be used in later chapters to search for QTL. The data generated in this experiment represent the most extensive metabolic dataset available internationally for pregnant dairy heifers and accordingly offer a unique opportunity to examine the relationship between metabolic phenotypes and genetic markers.

In the current study animals were not housed nor fed a formulated diet. Therefore, environmental variables such as ambient temperature, day light hours and quality and quantity of feed the animals consumed were likely to vary between groups. Adjustment for the effects of these environmental factors on the blood metabolites and hormones provided a more accurate data set for use in generating phenotypic variables that were used in QTL searches.

In this chapter, the response curves for each of the challenges are presented and the important features of the curves are described. The impact of environmental variables is then discussed and the models used for adjusting the data are presented. Finally the metabolic phenotypes calculated for use in the QTL searches are explained.

4.2 Mean response curves

A description of the parameters measured in response to the glucose, adrenaline and TRH challenges is given in this section. The mean curve of each of the response parameters is given, followed by a discussion of the factors that were found to influence each of the response variables.

4.3 Adrenaline challenge

Adipose tissue is a vital energy source during lactation in the dairy cow. During early lactation the NEFA released by adipose tissue contribute directly and substantially to milk fat secretion and to oxidative energy metabolism in different tissues, in doing so, spare glucose and amino

acids for use by the mammary gland (Chilliard *et al.* 2000). Between 20 to 43% of a cow's lipids may be mobilised during the first two months of lactation to supply the energy demands imposed by milk synthesis (Chilliard *et al.* 1984, Chilliard *et al.* 1991a). The extent of mobilisation is dependent on factors such as milk production, body condition, age of the cow, parity and diet (Komaragiri and Erdman 1997, McNamara 1994). Selection based on increased milk yield has a correlated response in feed intake that only covers about one-half of the extra energy requirements for the increase in yield. Hence, energy balance progressively decreases with increasing genetic merit for yield and this energy gap is most likely filled by changes in energy partitioning and fat mobilisation (Veerkamp *et al.* 2003). Therefore, the ability of an animal to mobilise adipose tissue for this purpose may affect her ability to sustain high levels of milk production, particularly during early lactation when feed intake does not increase as rapidly as the energy requirements.

Catecholamines are among the most effective regulators of adipose tissue lipolysis, which is stimulated through the β-adrenoreceptors (β-AR) and inhibited through α2-AR (Lafontan 1994). The major storage form of lipid is triacylglycerol which yields NEFA and glycerol when lipolysis occurs (Dunshea *et al.* 1990). Adipose tissue lipolytic potential can be estimated *in vivo* by plasma glycerol or NEFA response to injection or infusion of catecholamines (Chilliard *et al.* 1998b, Kolver *et al.* 2001, McCutcheon and Bauman 1986, Xing *et al.* 1991). Changes in plasma NEFA concentrations in response to an adrenaline or fasting challenges were not different between high and low selection lines (Mackenzie *et al.* 1988, Michel *et al.* 1991, Xing *et al.* 1991). However, a significant ($P<0.01$) linear relationship was found between daily milk energy secretion and NEFA area response to an adrenaline challenge, the regression accounted for 67% of the variation in milk energy output (McCutcheon and Bauman 1986). Animals of higher genetic merit for milk production have consistently higher rates of lipolysis and hormone sensitive lipase activity (McNamara 1994). NEFA liberated during lipolysis can be released into the circulation or re-esterified into newly formed triacylglycerols. Conversely, glycerol kinase is absent from bovine adipose tissue which means reutilisation of glycerol for

triacylglycerol synthesis is negligible (Metz *et al.* 1973) and glycerol released during lipolysis must enter the circulation (Dunshea *et al.* 1990). Plasma glycerol concentrations in response to an adrenaline challenge more accurately reflect lipolysis as opposed to plasma NEFA which reflects lipolysis and re-esterification (Kolver *et al.* 2001, McNamara and Hillers 1986a). Plasma NEFA, glycerol and glucose concentrations measured in response to an adrenaline challenge will provide information on the ability of animals to mobilise triacylglycerol from adipose tissue and glycogen from the liver.

Results

Baseline plasma glycerol concentrations increased with time, in the pre-challenge samples (Figure 4.1). After adrenaline was infused plasma glycerol concentrations increased rapidly with maxima reached 4 minutes after challenge in 60% of heifers, and 90% within 15 minutes. In all heifers there was a steady decline in plasma glycerol concentrations after maximum concentrations were reached, however only 26% of heifers had returned to the concentration measured at the -20 minute sample time, by the end of the sampling period, 90 minutes after the challenge. There was large variation between heifers in the magnitude of their glycerol response to the adrenaline challenge as shown by the large standard deviations. This is likely to be due to differences between animals in triacylglycerol mobilisation in response to the adrenaline challenge and the speed at which glycerol was cleared from the circulation.

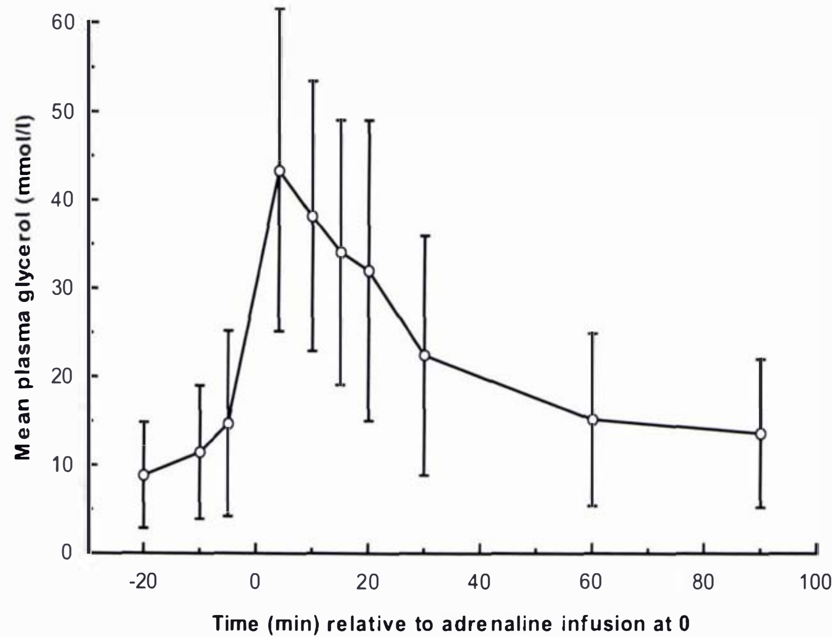


Figure 4.1 Mean plasma glycerol concentrations before and after an intravenous adrenaline injection (1.4 μg adrenaline/kg lwt) at time zero. Vertical bars display standard deviation.

Year had an effect on plasma glycerol concentrations at -5, 4 and 20-60 minutes ($P<0.001$) relative to adrenaline infusion (Figure 4.2, left panel). Sire had an effect on glycerol concentrations at all time points ($P<0.001$) (Figure 4.2, right panel). A table with the adrenaline challenge response variables measured, and the significance of the fixed effects on each of these response variables can be found in Appendix D, Table 9.2.

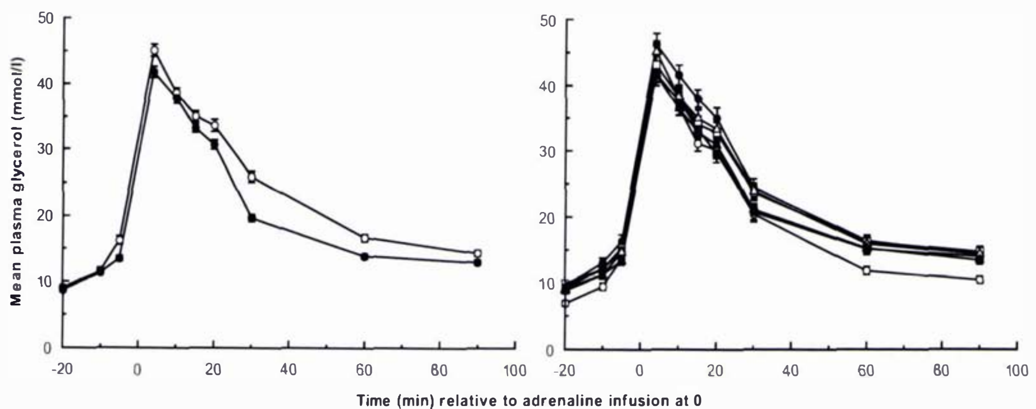


Figure 4.2 Mean plasma glycerol concentrations, by challenge year (left panel; ○ 2002; ● 2003) and by sire (right panel; ○ 14415740; ● 14915827; □ 15525685; ■ 15656114; △ 16109405; ▲ 16120004), before and after an intravenous adrenaline injection (1.4 μg adrenaline/kg lwt) at time zero. Vertical bars display standard errors at each time.

Plasma NEFA concentrations were increasing over time in the pre-challenge samples (Figure 4.3). After the adrenaline challenge was given at time zero, plasma NEFA concentrations continued to increase. Ninety percent of heifers reached maximum plasma NEFA concentrations between 4 and 20 minutes after the challenge, with 58% of heifers between 15 and 20 minutes.

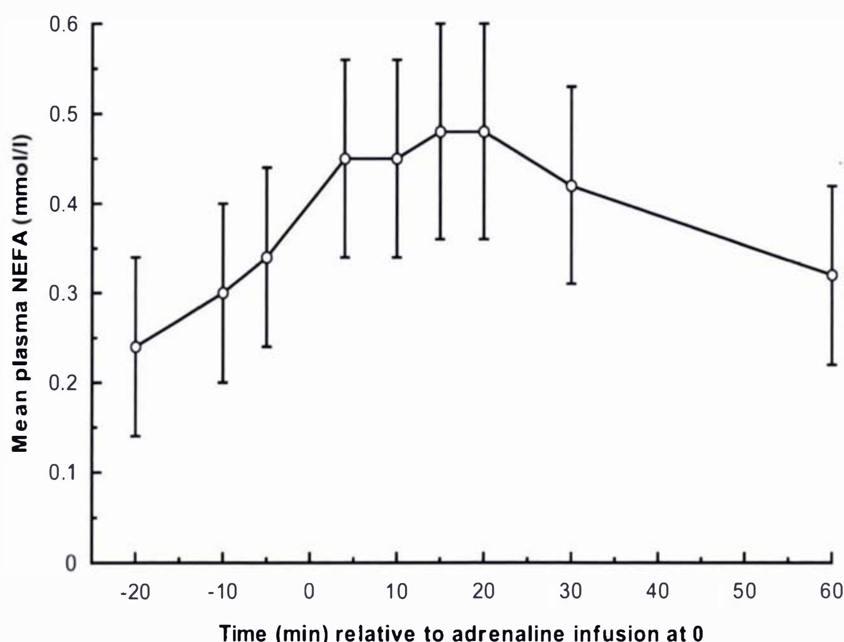


Figure 4.3 Mean plasma NEFA concentrations before and after an intravenous adrenaline injection (1.4 μg adrenaline/kg lwt) at time zero. Vertical bars display standard deviations.

In general, plasma glucose concentrations increased and reached maximum by 10 to 30 minutes after the adrenaline injection (Figure 4.4). Once maximum was reached, there was a steady decline in plasma glucose concentrations, 57% of heifers had returned to baseline plasma glucose concentration by 120 minutes post adrenaline injection.

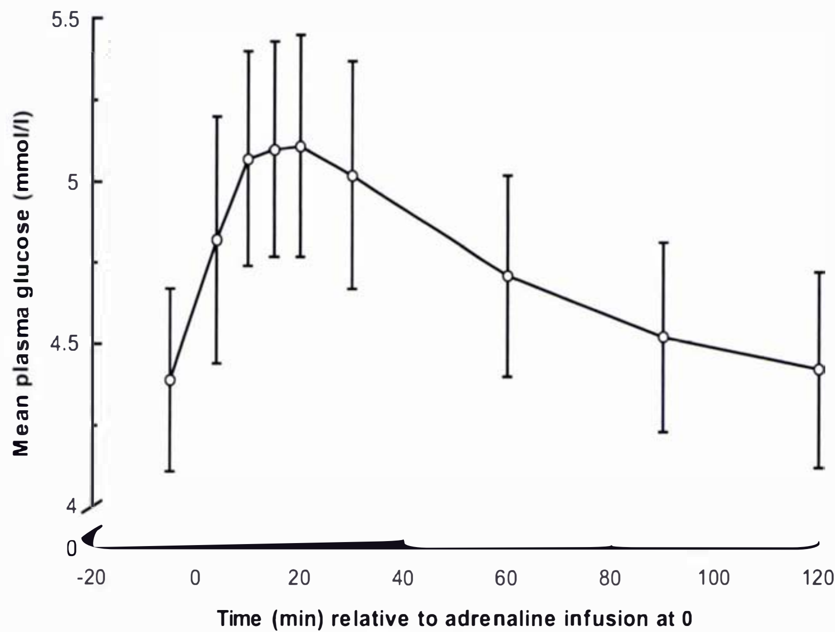


Figure 4.4 Mean plasma glucose concentrations before and after an intravenous adrenaline injection (1.4 μg adreanline/kg lwt) at time zero. Vertical bars display standard deviations.

Discussion

The enhanced plasma concentrations of NEFA after an adrenaline challenge represent the mobilisation of fatty acids, that is, the net effect of β-induced increased lipolysis and fatty acid re-esterification, and the α2-induced opposition of these events (Houseknecht *et al.* 1995, Kolver *et al.* 2001). The response in plasma glycerol concentrations to an adrenaline challenge reflects lipolysis, hormone sensitive lipase activity, as adipose tissue does not reutilise glycerol due to very low glycerol kinase activity (Kolver *et al.* 2001, McNamara and Hillers 1986a). Furthermore, the approximate 4.5-fold increase in glycerol concentration in response to an adrenaline challenge is considerably greater than the 2-fold increase in NEFA concentration suggesting significant re-esterification of NEFA in adipose tissue or a more rapid removal of NEFA than glycerol from the circulation.

The higher concentrations of glycerol after the adrenaline injection in cohort 1, in comparison with cohort 2, are consistent with the higher basal GH concentrations in cohort 1 (Figure 4.14). GH treatment is known to increase the sensitivity of the adipose tissue to adrenaline in lactating

dairy cows (Houseknecht *et al.* 1995, McCutcheon and Bauman 1986, Sechen *et al.* 1989) and in growing steers (Peters 1986). GH may alter lipolysis centres on the G inhibitory system of adipose tissue. Thus the enhanced lipolytic response to catecholamines observed *in vivo* in GH-treated animals is in large part related to a relief in the tonic inhibition of lipolysis via changes in the G_i signalling cascade (Etherton and Bauman 1998) which ultimately results in activation of hormone-sensitive lipase (Lafontan 1994, McNamara 1994). Hormone-sensitive lipase catalyses the rate limiting reaction in lipoplysis, initial hydrolysis of triacylglycerol to form diacylglycerols (Beitz and Nizzi 1997).

The significant effect of sire on glycerol response to adrenaline challenge indicates genetic control of adipose tissue β-adrenoreceptor responsiveness of one or possibly several points of the cascade initiated by catecholamine binding to β-adrenergic receptors through to phosphorylation and activation of hormone sensitive lipase (McNamara 1994). Lipid mobilisation is vitally important in supplying the energy demands of lactation in the dairy cow, particularly in early lactation when feed intake does not increase as rapidly as feed demand (McNamara 1995). High milk yields in dairy cows are related to the ability to mobilise body energy reserves in early lactation (Bauman *et al.* 1985). As mentioned previously, a linear regression between daily milk energy secretion and NEFA area response to an adrenaline challenge accounted for 67% of the variation in milk energy output (McCutcheon and Bauman 1986). In addition, animals of higher genetic merit for milk production have consistently higher rates of lipolysis and hormone sensitivity lipase activity (McNamara 1994). Therefore, identifying genetic markers in hormonal control and metabolic pathways of adipose tissue mobilisation may well be related to QTL for milk production, particularly milk fat yield.

4.4 Glucose challenge

High producing dairy cows are in negative energy balance during early lactation, because dietary supply of nutrients is insufficient to meet the nutrient requirements for maintenance and milk synthesis (Bell 1995, de Vries and Veerkamp 2000, Van den Top *et al.* 2005). In this period the nutrient and energy supply to the mammary gland is strongly dependent on the cow's

ability to mobilise body reserves and possibly the ability to reduce the requirements of peripheral tissues (Sejrsen *et al.* 1984, Vernon 1998, Vernon and Pond 1997). Changes in nutrient use by peripheral tissues is partly facilitated by altered concentrations of metabolic regulatory hormones such as insulin and GH (Sartin *et al.* 1988). Insulin is the dominant hormonal regulator of the energy metabolism in resting ruminants (Brockman 1986). Plasma insulin concentrations are low in early lactation (Lukes *et al.* 1989) promoting greater gluconeogenesis in the liver and reduced use of nutrients by peripheral tissues (Brockman 1986). Thus, differences between dairy cows in the way glucose and insulin metabolism are affected at the onset of lactation is likely to influence their ability to produce milk.

The concentration of insulin was found to be higher in the low- (Hereford × Friesian) than in the high-yielding (Friesian) group during lactation (Hart *et al.* 1978). Also, plasma NEFA concentrations were significantly higher in the high- compared to low-yielding cattle (Hart *et al.* 1978). These findings were followed by numerous investigations into the relationship between plasma insulin concentrations and milk yield with highly inconsistent results (Woolliams and Løvendahl 1991). Differences between high and low milk yield selection lines in glucose and insulin metabolism have been reported in a number of studies (Mackenzie *et al.* 1988, Min *et al.* 1993, Xing *et al.* 1993). But these differences have not been present or were reversed in other studies (Barnes *et al.* 1985, Lukes *et al.* 1989, Osmond *et al.* 1981), however, in some cases this may have been due to experimental design, i.e. measurement of steady-state conditions rather than perturbing the system.

The intravenous injection of glucose is a well established physiological challenge for assessing the responsiveness of the pancreas and its ability to release insulin as well as the rate of tissue uptake of glucose (Åkerlind *et al.* 1999, Kaneko 1997, McCann *et al.* 1986). Investigations in non-lactating dairy cattle indicate an association between genetic improvement and changes in insulin secretion in response to a glucose challenge (Mackenzie *et al.* 1988). It is thought that lactating cows, which partition nutrients away from the adipose tissue and toward mammary gland are insulin resistant because there is a decrease in the sensitivity of their adipose and

muscle tissues to insulin (Cronjé 2000). Consequently, glucose uptake by adipose and muscle tissue in response to insulin is less in lactating than non-lactating cows. This is a modification of a metabolic process in response to a change in physiological state that can be detected by a glucose challenge. Thus, it is postulated that there may be differences even between non-lactating animals in their response to a glucose challenge, which may indicate differences in the alleles they possess for key genes in energy metabolism and nutrient partitioning.

Results

Administration of glucose caused a rapid increase in blood glucose concentration with a mean peak plasma glucose concentration of 19.4 mmol/l (Figure 4.5). All heifers achieved peak glucose concentration by 2 minutes after the completion of glucose infusion. Following the peak, there was a rapid decline in glucose concentration, however, only 11% of heifers tested had reached basal concentration by 90 minutes after challenge.

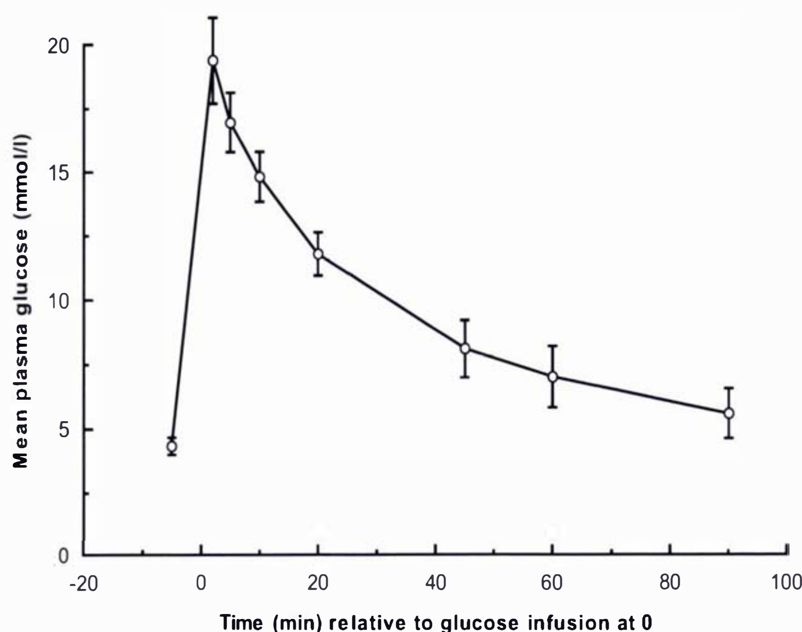


Figure 4.5 Mean glucose concentrations in the plasma of heifers, before and after an intravenous glucose infusion (0.3 g glucose/kg lwt) at time zero. Vertical bars display standard deviation at each time.

In general, there was an immediate increase in plasma insulin concentration following the glucose infusion. Maximum plasma insulin concentrations were reached between 10 to 20 minutes after glucose infusion, for 99% of the heifers tested, followed by a period of steady decline. Concentrations of insulin remained above baseline concentration during the 60 minutes after the glucose challenge.

There was large variation between heifers in the magnitude of their insulin response to the glucose challenge as shown by the large standard deviations (Figure 4.6). In addition, while there was little variation in peak plasma glucose concentrations, insulin peak concentrations were markedly different between heifers.

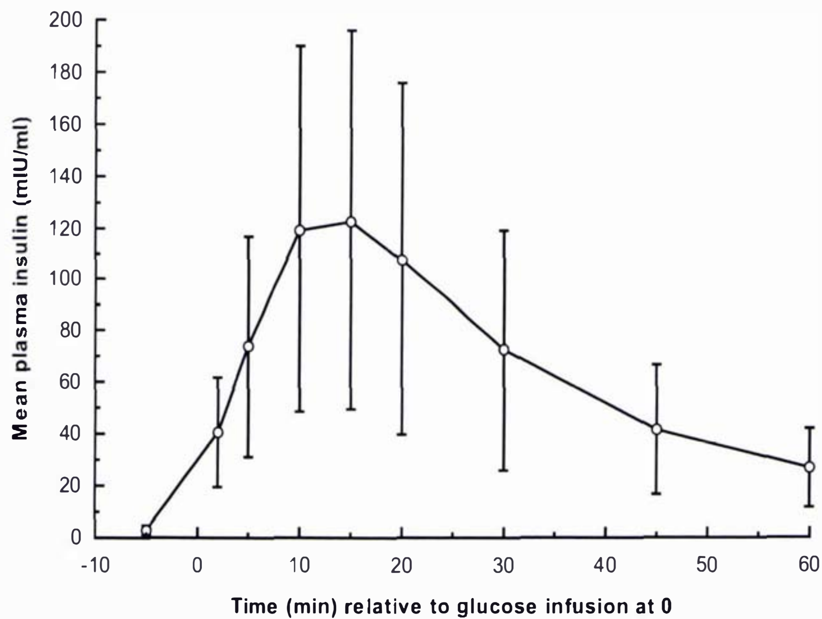


Figure 4.6 Mean insulin concentrations in the plasma of heifers, before and after an intravenous glucose infusion (0.3 g glucose/kg lwt) at time zero. Vertical bars display standard deviation at each time.

The plot of fractional turnover rate of glucose against insulin maximum (Figure 4.7) shows a weak positive relationship between peak plasma glucose concentration and insulin peak concentration.

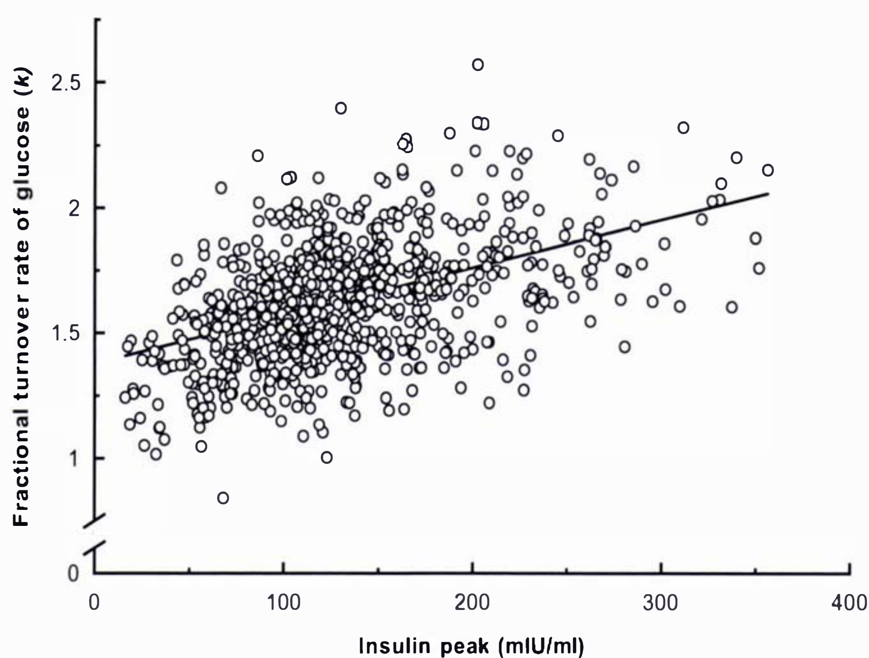


Figure 4.7 Linear regression between glucose clearance and insulin peak, Y = 0.0019X + 1.3779, R^2 = 0.2224

There was a significant effect of year, group nested within year, and sire ($P<0.001$) on plasma insulin concentration at all time points measured (Figure 4.8). Figure 4.8 shows the mean insulin concentrations calculated from all heifers challenged in each of the challenge years (left panel). Figure 4.8, right panel, shows the mean insulin concentrations of all heifers challenged in both years grouped by the six sires used in this experiment. Maternal grandsire also had a significant effect on plasma insulin concentrations, 2-45 minutes ($P<0.001$), 60 minutes ($P<0.01$) and -5 minutes ($P<0.05$) relative to the time of challenge.

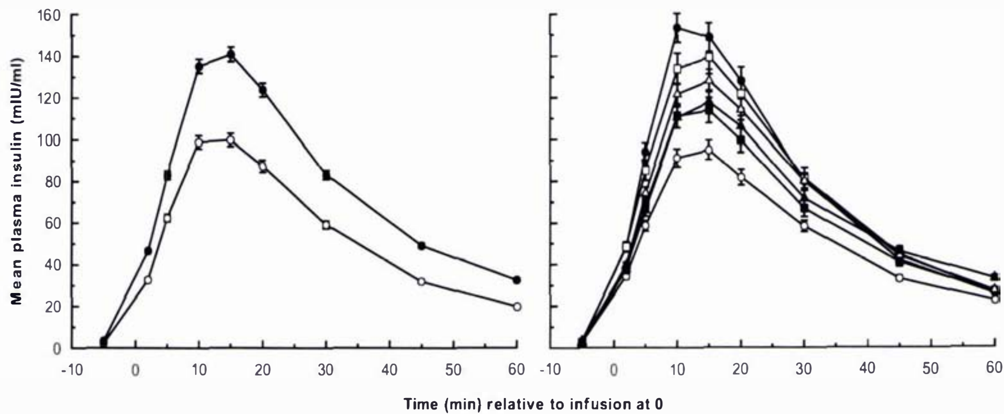


Figure 4.8 Mean insulin concentrations in the plasma by challenge year (left panel; ○ 2002; ● 2003) and by sire (right panel; ○ 14415740; ● 14915827; □ 15525685; ■ 15656114; △ 16109405; ▲ 16120004), before and after an intravenous glucose infusion (0.3 g glucose/kg lwt) at time zero. Vertical bars display standard errors at each time.

There was also a significant effect of year, group nested within year, and sire ($P<0.05$) on plasma glucose concentration at all time points measured, with the exception that sire did not have a significant affect on plasma glucose concentration at the -5 minute time point (Figure 4.9). The differences are small so are difficult to see but they are significant, this is likely to be due to the small variation in plasma glucose concentrations and thus very small standard errors.

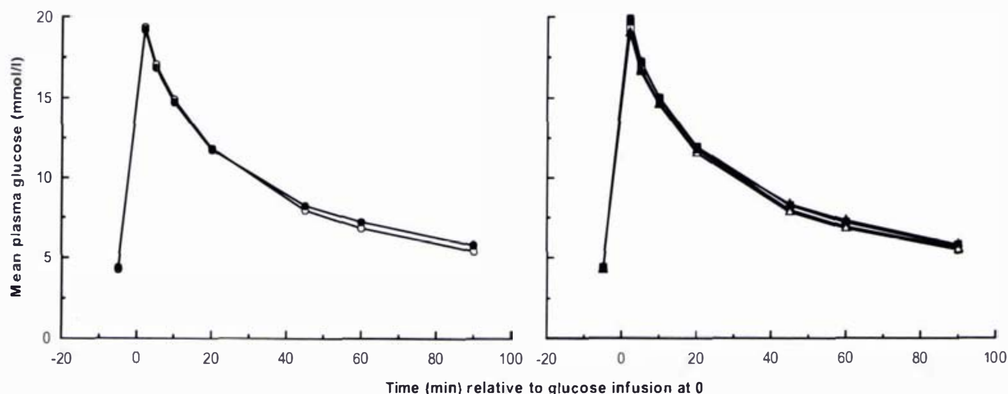


Figure 4.9 Mean glucose concentrations in the plasma by challenge year (left panel; ○ 2002; ● 2003) and by sire (right panel; ○ 14415740; ● 14915827; □ 15525685; ■ 15656114; △ 16109405; ▲ 16120004), before and after an intravenous glucose infusion (0.3 g glucose/kg lwt) at time zero. Standard error bars were drawn on both graphs but are too small to be seen.

Baseline NEFA concentrations were increasing prior to the infusion of glucose and continued to increase up to 15 minutes post infusion (Figure 4.10). The maximum NEFA concentrations

occurred between -5 and 10 minutes relative to glucose infusion, in 89% of the heifers, with the greatest proportion (34% of heifers) at 5 minutes after glucose infusion. Following the maximum NEFA concentration, there was a rapid decline in NEFA concentration until a nadir was reached at 60 or 90 minutes after glucose infusion, in 87% of heifers. Once the nadir was reached, plasma NEFA concentration began to increase.

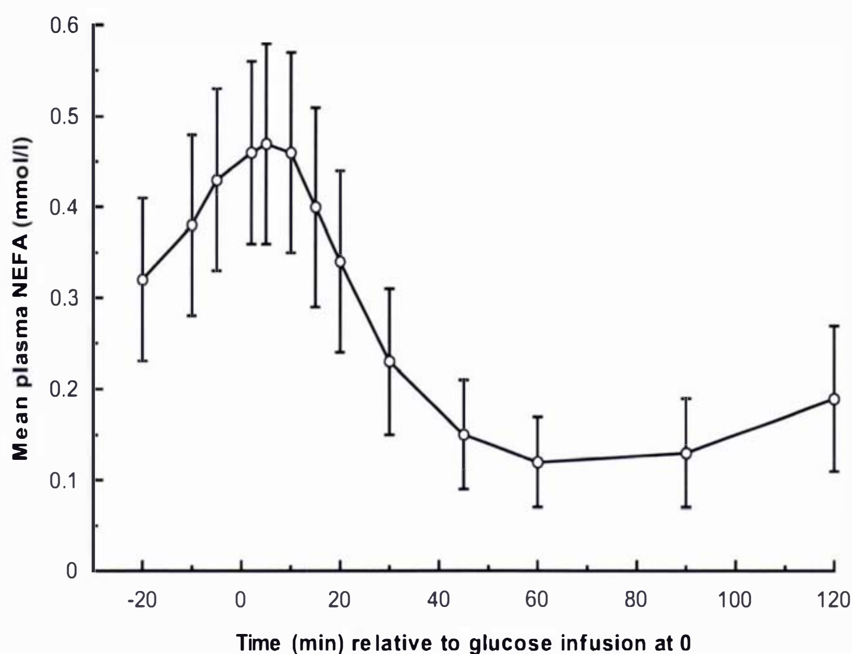


Figure 4.10 Mean NEFA concentrations in the plasma of heifers, before and after an intravenous glucose infusion (0.3 g glucose/kg lwt) at time zero. Vertical bars display standard deviation at each time.

There was a significant effect of sire and group, nested within year, (P<0.001) on plasma NEFA concentration at all time points measured. Maternal grandsire had a significant effect on plasma NEFA concentrations at all time points except at 60 and 90 minutes and the effect of year was only significant at -10 to -5 minutes relative to time of challenge (P<0.001).

Analysis of the insulin data showed that the mean response to the glucose challenge varied markedly between groups. There was large variation between groups in mean insulin response at 10 minutes after the glucose infusion, despite the reasonably consistent mean glucose peak across groups (Figure 4.11). The peak glucose concentration will be reflective of the glucose

distribution space, which space is defined as the volume of extracellular fluid in which the glucose pool is distributed (Feller *et al.* 1950). The glucose distribution space will be relatively constant between animals, therefore the relatively small variation in plasma glucose concentrations is expected.

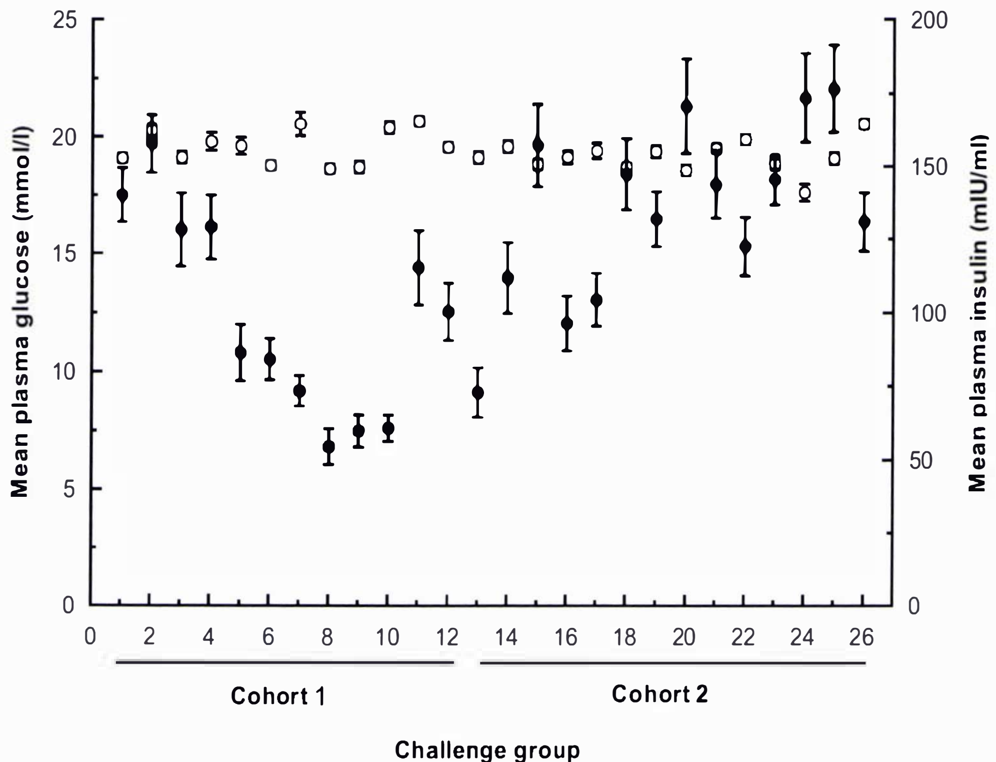


Figure 4.11 Mean plasma insulin concentrations (●, insulin mean for plasma samples 10 minutes after challenge) and peak concentrations of glucose in plasma (○, glucose mean for plasma samples 2 minutes after challenge) in response to an intravenous glucose challenge (0.3 g glucose/kg lwt). Cohort 1 is challenge groups 1 to 12 and Cohort 2 is challenge groups 13 to 26

In contrast, the insulin concentration will reflect the sensitivity of the pancreas to glucose, which is known to vary between animals and their physiological state. There is considerable variation between group means of the 10 minute insulin samples from cohort 2 as well as cohort 1, however, the major concern was the consistently low insulin concentrations in groups 5 to 10 (Figure 4.11). The challenge groups were balanced for sire, live weight and date of birth, therefore it would seem unlikely that all of the animals that have low insulin release in response to a glucose challenge would end up in the same groups. It is more likely that there is a

systemic error or an event that has affected these groups causing the animals in these groups to have consistently low insulin responses.

To test if a systemic error had occurred, a stratified subset of samples (208, 10 minute plasma samples, 8 per challenge group) were selected and re-analysed. The regression equations for the original results, regressed against the re-analysed results (Figure 4.12), had R^2 values of 0.941 and 0.9662 for the cohort 1 and 2 samples, respectively. The respective regression coefficients indicate that for every 1 mIU/ml of insulin in the original analyses there were 0.936 mIU/ml and 0.81 mIU/ml of insulin in the re-analyses for cohort 1 and 2 samples, respectively.

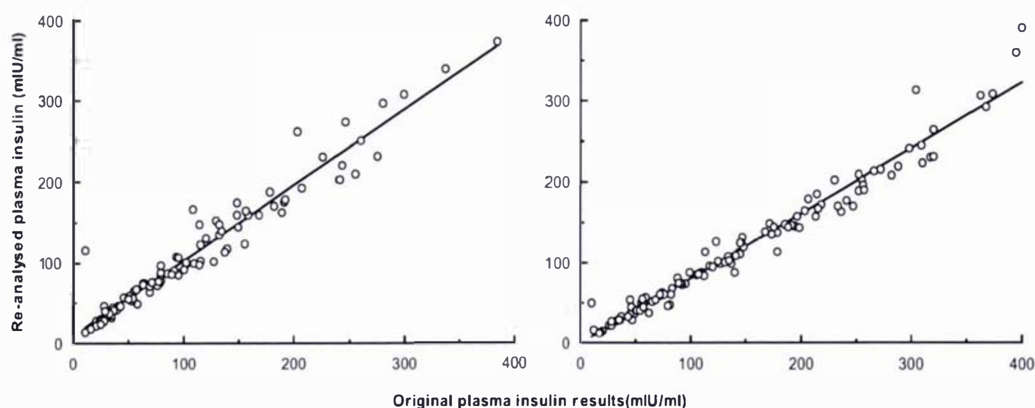


Figure 4.12 Regression between a subset of original and re-analysed plasma insulin samples taken 10 minutes post glucose infusion. Cohort 1, (left panel) $Y = 0.9361x + 9.3865$. $R^2 = 0.941$; Cohort 2, (right panel) $Y = 0.8098x - 0.3579$ $R^2 = 0.9662$

Insulin concentrations in bovine serum samples have been reported to be stable when stored at -20 °C for 8 days (Reimers *et al.* 1983). Thus, it would seem likely the downward drift in the results from the original analyses to when the subset was re-analysed, is due to differences in calibration of the Immulite® (Diagnostic Products Corporation, Los Angeles, CA, USA) between the original and re- analyses and this difference between original and re-analyses calibration was greater for cohort 2 samples. The large number of samples, the length of time it would take and considerable cost of reanalysing more than 7700 plasma samples meant that the decision was made not to re-run the insulin assay on all the plasma samples. Instead, data were adjusted statistically for the effect of group.

All environmental variables tested had a significant effect on baseline insulin concentrations (Table 4.1). However, the 5-day average (four days preceding plus the day of challenge) for minimum daily temperature and for daily rainfall were the only two environmental variables that had a significant effect on insulin concentration after the glucose challenge was given, from 2 to 45 minutes post infusion.

Table 4.1 The significance of the effect of fixed and environmental parameters on insulin concentrations 5 minutes before and successive times after the intravenous infusion of glucose (0.3 g glucose/kg lwt) in dairy heifers (⋆ 0.05 to 0.01, ⋆⋆ 0.01 to 0.001, ⋆less than 0.001). MGS – maternal grandsire; THI – temperature, humidity index

	-5	2	5	10	15	20	30	45	60
Year	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆
Sire	⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆
MGS	⋆⋆	⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆	⋆⋆⋆	⋆⋆	⋆⋆⋆	⋆⋆
Max temp	⋆⋆⋆	NS	NS	NS	NS	NS	NS	NS	NS
Max temp 5d ave	⋆⋆⋆	NS	NS	NS	NS	⋆	NS	NS	NS
Min temp	⋆⋆⋆	NS	NS	NS	NS	NS	NS	NS	NS
Min temp 5d ave	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆	NS
Rain 5d ave	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆	NS
THI	⋆⋆⋆	NS	NS	NS	NS	NS	NS	NS	NS
R^2	0.325	0.35	0.309	0.327	0.315	0.303	0.264	0.281	0.288

Group was fitted in the model, nested within year, the R^2 values (Table 4.2), instead of the environmental variables in Table 4.1. At all time points were higher than the R^2 values when then environmental variables were fitted in the model (Table 4.1).

Table 4.2 The significance of the effect of fixed parameters on insulin concentrations 5 minutes before and successive times after the intravenous infusion of glucose (0.3 g glucose/kg lwt) in dairy heifers (⋆ 0.05 to 0.01, ⋆⋆ 0.01 to 0.001, ⋆less than 0.001). MGS – maternal grandsire

	-5	2	5	10	15	20	30	45	60
Year	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆
Sire	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆
MGS	⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆
Group(year)	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆	⋆⋆⋆
R^2	0.37	0.387	0.373	0.39	0.371	0.367	0.332	0.337	0.343

Discussion

These glucose results are consistent with another experiment that found that basal concentrations of glucose in heifers were not reached until 120 minutes after a glucose challenge (Xing *et al.* 1991). The weak relationship between peak plasma glucose peak plasma insulin peak concentrations (Figure 4.7) may indicate that some heifers are more responsive to a glucose challenge than others, that is, the amount of insulin released in response to the glucose challenge may vary between heifers possibly due to differences in each animal's sensitivity and or responsiveness to the glucose stimulus. Non-insulin mediated glucose uptake (NIMGU) accounted for 62.5% of glucose utilisation when insulin concentrations were elevated above basal in dairy cows in mid-lactation (Rose *et al.* 1997). However, 82-88% of NIMGU was attributed to glucose uptake by mammary gland (Rose *et al.* 1997), which would be a much smaller proportion in non-lactating heifers. Nevertheless, NIMGU may still differ between the heifers in this experiment, and may be another factor causing variation in the relationship between fractional turnover of glucose and insulin peak.

Some of the difference in plasma insulin concentrations between the two challenge years (left panel, Figure 4.8) can be attributed to the analytical error that has occurred in the insulin immunoenzymometric assay. However, the downward drift in plasma insulin concentrations in groups 5 to 10 in the 2002 challenge year (Figure 4.11) is not due to analytical error but rather is likely to be due to one, or a number of factors. These factors include environmental factors such maximum and minimum ambient temperature, relative humidity, rainfall and day length. Pasture quality in the time leading up to the challenge may have influenced plane of nutrition of the heifers, and plane of nutrition may influence plasma insulin concentration in response to a glucose challenge.

Basal insulin and glucose concentrations, in early-pregnant, non-lactating, Holstein-Friesian cows, were found to be significantly lower in a hot compared to a thermoneutral environment (Itoh *et al.* 1998). Peak insulin concentrations after glucose infusion were significantly lower during exposure to high temperatures (Itoh *et al.* 1998). It was suggested that lower basal and

peak plasma insulin concentrations in response to glucose infusion during heat exposure was due to a lower insulin secretory response. However, there was no difference in insulin AUC between thermal environments (Itoh *et al.* 1998) which may indicate that the actual secretion of insulin in response to a glucose challenge was the same, there was no evidence of a more sustained release of insulin which would affect AUC, the peak was only lower in the hot environment due to the lower basal concentration.

In the current study, plasma insulin concentration in the 10 minute post infusion sample and the insulin AUC were highly correlated (0.93, P<.0001). Consequently, the group means for the insulin AUC had a very similar pattern to the group means of the 10 minute plasma concentrations (Figure 4.11). In addition, the mean baseline concentrations also showed a similar pattern. Thus, the group effect is consistent across time points, both pre- and post-glucose infusion, and is still apparent for AUC. It appears that the factor causing lower plasma insulin concentrations in groups 5-10 may be reducing both the basal concentrations and the glucose stimulated insulin secretory response.

It has been reported that a temperature-humidity index (THI) based on the minimum daily temperature was more closely correlated with dry matter intake than was the THI based on the maximum daily temperature (Holter *et al.* 1996). Heat stress does not cause such a dramatic reduction in feed intake of cows if they are exposed to a minimum daily temperature of less than 10° C for at least 4 hours per day as this allows dissipation of thermal heat loads (Roseler *et al.* 1993). This is consistent with the current study results (Table 4.1), where the average minimum temperature had a significant effect on plasma insulin concentration up to 45 minutes after the challenge. The range in average minimum temperature was 6.4-16 °C. Average maximum temperature only had a significant effect on baseline insulin and at 20 minutes after glucose challenge. In lactating cows, milk yield was found to be significantly correlated with weather conditions 2, 3 and 4 days previous but not on the same day as the milk yield measurement (Maust *et al.* 1972). Reduction in milk yield was thought to be related to a rise in body temperature causing a depression in feed intake (Maust *et al.* 1972) and elevated maintenance

energy requirements as a consequence of cows attempting to lose body heat (Roseler *et al.* 1993). It is possible that there was a similar effect of ambient temperature, in the days prior to the day of challenge, on plasma insulin concentration in the pregnant heifers in this trial, which is why the 5 day average minimum temperature was significant rather than the minimum temperature on the day of challenge.

High feeding levels have been shown to increase plasma insulin concentrations compared with low feeding levels in prepubertal and 6-8 month old heifers (Stelwagen and Grieve 1992, Vestergaard *et al.* 2003, Xing *et al.* 1991). In addition, both energy and nitrogen content of the diet have been demonstrated to have a significant affect on plasma insulin concentration in Romney wether sheep (Waghorn *et al.* 1987). It is possible that pasture available to groups 5 to 10 was of a lower quality and quantity, causing lower plasma insulin concentrations at baseline and in response to glucose challenge, than in other challenge groups. January and February are summer months in New Zealand and usually coincide with low rainfall and high evapo-transpiration rates (evaporation from soil plus transpiration by plants), which may cause reduced pasture production and quality (McKenzie *et al.* 1999). During summer there is often a high proportion of mature pasture in the sward. Mature pasture has a high fibre content and a reduced proportion of protein and readily digestible carbohydrate. Thus, energy and protein intake are often limited from summer pasture compared with pasture at other times of the year (Hodgson and Brookes 1999).

Since pasture samples were not taken on a regular basis during the challenge periods, the 5 day average daily rainfall (DADR) variable was included in the model. It was included as an indirect indicator of feed quality leading up to the challenge because rainfall promotes pasture growth and hence influences pasture quality. DADR may have had a significant effect on plasma insulin concentration both pre- and post-glucose infusion (-5 to 45 minutes) either because of its effect on pasture quality or because higher rainfall reduced heat stress.

Plasma IGF-I concentrations have also been shown to be related to feed intake, when feed intake is low IGF-I concentrations are also low compared with those of animals on high feed intake (Breier *et al.* 1986, Ronge and Blum 1989). If plasma insulin concentrations were low in challenge groups 5-10 of the current study, their plasma IGF-I concentrations could also be expected to be low. The Pearson correlation coefficient between baseline insulin and mean IGF-I concentrations in plasma while significant ($P<0.0001$), is weak (R=0.15), which suggests there are other factors, in addition to energy intake, that are influencing insulin concentration. In addition, the plasma IGF-I concentrations used for this correlation were measured the day after the glucose challenge, following overnight re-feeding. This is likely to reduce the strength of the relationship between plasma IGF-I and insulin concentrations. Even so, animals in groups 5-10 may still have experienced a lower level of nutrition than other challenge groups but it not sufficient to reduce plasma IGF-I concentrations. The finding that only the animals fed to below maintenance had significantly reduced plasma IGF-I concentrations compared with the high and medium feeding level groups (Breier *et al.* 1986), is consistent with this theory.

The environmental variables tested in the model (Table 4.1) were only measured at one weather station site. Therefore, the same data were applied to all animals in one challenge group, as a result, of these data were confounded within group. When group was fitted in the model, nested within year, the R^2 values (Table 4.2), at all time points were higher than the R^2 values when then environmental variables were fitted in the model (Table 4.1). Thus, fitting group nested within year explained more of the variation in insulin concentration than was explained by fitting the environmental variables. It is likely there are other variables which affected insulin concentration and were confounded within group that were not measured, therefore, by fitting group in the model all of these variables both unknown and known were adjusted for.

Future studies that involve metabolic challenges should include collection of a complete set of data for quantifying environmental conditions (Woolliams and Løvendahl 1991). This could be achieved by taking daily pasture samples to try and account for the influence of feed intake and plane of nutrition on responses to a glucose challenge, IGF-I should be measured at the same

time as insulin and regular ambient temperature measurements should be recorded. Ideally, such experiments would be conducted under controlled environment conditions where, lighting, ambient temperature, feed quality and intake could all be closely monitored and controlled but in this trial when a large number of animals were challenged this was not feasible. For the purpose of this study, group nested within year will be fitted in the model.

A number of metabolic phenotypes were calculated using the data from the glucose challenge. The objective was to calculate metabolic phenotypes that best described the metabolic events that occurred, and resulted in the response curves observed (section 4.7).

4.5 Thyrotropin-releasing hormone challenge

Administration of exogenous TRH has resulted in an increase in plasma GH, TSH and prolactin in dairy cattle (Hodate *et al.* 1985, Lacasse *et al.* 1991, McCutcheon and Bauman 1986). All three of these hormones are recognised as being important in lactation (Akers 2002). Plasma GH increases with the onset of lactation (Accorsi *et al.* 2005, Bell *et al.* 2000, Rhoads *et al.* 2004) and it appears to be antagonistic to the action of insulin thus preventing glucose uptake in insulin sensitive tissues and directing nutrients towards the mammary gland (McDowell *et al.* 1987, Rose *et al.* 1996, Vernon and Finley 1988). Basal or artificially stimulated concentrations of GH in peripheral blood are greater in both adult and juvenile dairy cattle selected for high milk yield or yield of a milk component, for example milk fat, compared with average or low yielding selection lines (Beerepoot *et al.* 1991, Kazmer *et al.* 1986, Løvendahl and Sejrsen 1993, Løvendahl *et al.* 1991a, Lukes *et al.* 1989, Mackenzie *et al.* 1988, Roche *et al.* 2006b). Prolactin has a role in lactogenesis and is a primary stimulator of mammary cell differentiation in the dairy cow (Akers 2006, Tucker 2000). Animals selected for high milk production have been shown to have higher plasma prolactin concentrations around the time of parturition (Lukes *et al.* 1989) and it has been suggested that greater responsiveness and sensitivity to prolactin during transition to lactation may be associated with an increase in subsequent lactation (Auchtung *et al.* 2005).

Thyroid hormones are involved in metabolic homeostasis and maintenance of lactation (Perera *et al.* 1985). During early lactation in cows there is a decrease in T_4 and T_3 concentrations and a number of studies have found a negative relationship between milk yield and circulating concentrations of thyroid hormones (Hart *et al.* 1978, Nixon *et al.* 1988, Pezzi *et al.* 2003, Walsh *et al.* 1980). Consistent with this, there was a tendency for circulating T_4 concentrations during lactation to be lower in dairy cows selected for high milk yield (Bonczek *et al.* 1988, Hart *et al.* 1978). In addition, Joakimsen *et al.* (1971) reported a positive genetic association between daily T_4 degradation and genetic merit for fat-corrected milk yield in AI stud bulls of 3 dairy breeds.

Adipose tissue is particularly important during lactation when increased lipolysis and decreased rates of lipogenesis contribute to meeting the energy demands of milk synthesis (Bauman and Vernon 1993, Etherton and Bauman 1998). Studies have shown that treatment mid-lactation cows with exogenous GH caused greater plasma concentrations of NEFA and glycerol both at baseline and in response to an adrenaline challenge when compared with controls (Houseknecht *et al.* 1995, McCutcheon and Bauman 1986, Sechen *et al.* 1990). At maximal responsiveness to adrenaline challenge plasma NEFA concentrations were increased approximately 6-fold, whereas glycerol concentrations were increased approximately 3-fold. It has been suggested that GH treatment reduces adrenaline-stimulated rates of re-esterification of fatty acids in adipocytes and increases lipolysis (Sechen *et al.* 1990). Measurement of NEFA concentrations in this challenge may give an indication of the action of GH on adipose tissue. Measurement of GH, prolactin and TSH concentrations may give some indication of genetic potential for milk production.

Results

Maximum concentrations of GH were reached 2-15 minutes after TRH infusion, in 93% of heifers. A steady decline in GH concentration followed the maximum. There was large variation between heifers in their GH response to the TRH challenge as shown by the large standard deviations (Figure 4.13), particularly at 2 and 5 minutes after the challenge.

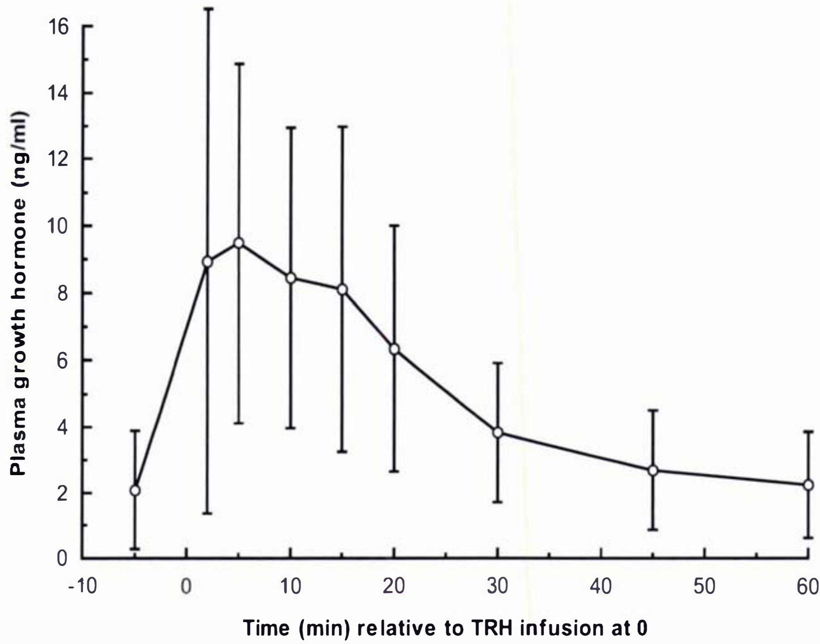


Figure 4.13 Mean GH concentrations in the plasma of heifers, before and after an intravenous TRH injection (0.3 μg TRH/kg lwt) at time zero. Vertical bars display standard deviation at each time.

There was a significant year effect on plasma GH concentration (P<0.001), except at the 10 minute sample time (Figure 4.14, left panel). Sire had a significant effect on plasma GH concentration (P<0.01) from 5 to 45 minutes after TRH infusion (right panel, Figure 4.14). A table with all the TRH challenge response variables measured, and the significance of the fixed effects on each of these response variables can be found in Appendix D.

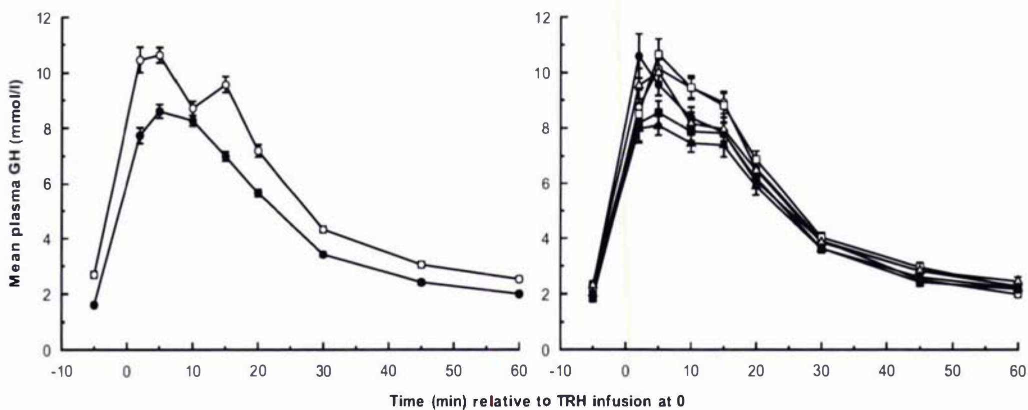


Figure 4.14 Mean plasma GH concentrations of all heifers, grouped by challenge year (left panel; ○ 2002; ● 2003) and by sire (right panel; ○ 14415740; ● 14915827; □ 15525685; ■ 15656114; △ 16109405; ▲ 16120004), before and after an intravenous TRH injection (0.3 μg TRH/kg lwt) at time zero. Vertical bars display standard errors.

Maximum concentrations of prolactin were reached between 2-10 minutes post TRH infusion (Figure 4.15), in 90% of heifers, followed by a rapid decline in prolactin concentrations. Approximately, 47% of heifers had plasma prolactin concentrations that reached baseline, by 150 minutes after the TRH infusion.

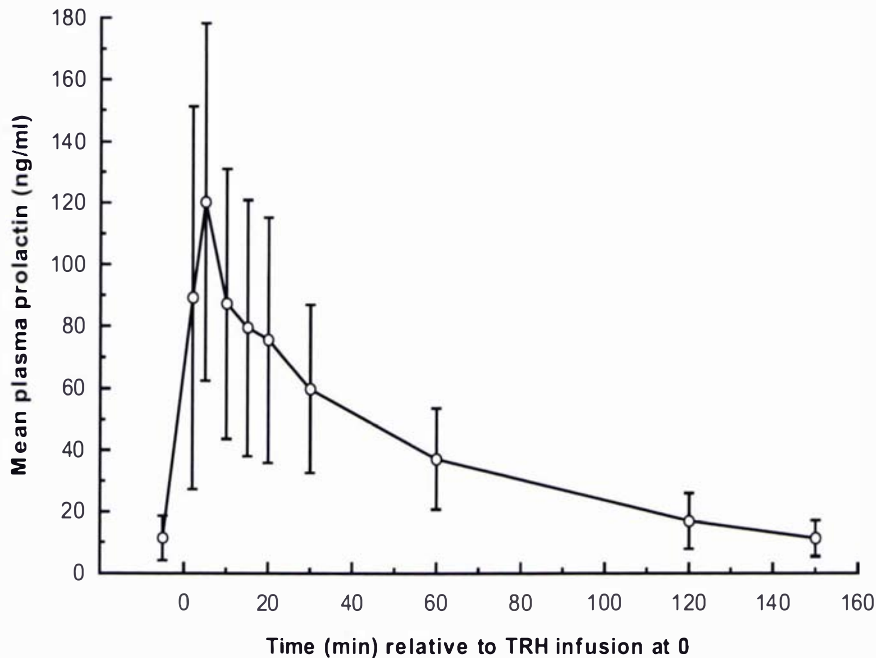


Figure 4.15 Mean prolactin concentrations in the plasma of heifers, before and after an intravenous TRH injection (0.3 μg TRH/kg lwt) at time zero. Vertical bars display standard deviations at each sample time.

Plasma prolactin concentrations were significantly correlated with day length ($P<0.001$) both before and after the TRH challenge, with the exception of the 2 minute plasma sample. Heifers exposed to longer days at the time of TRH challenge tended to have greater plasma prolactin concentrations than those exposed shorter days (Figure 4.16). This finding is consistent with controlled lighting studies that have shown heifers or cows exposed to a greater number of light hours have greater mean serum prolactin concentrations than heifers or cows exposed to fewer light hours (Auchtung *et al.* 2005, Newbold *et al.* 1991). Environmental variables were fitted in the model for correcting the challenge data but as with the glucose challenge data fitting a group, nested within year, effect explained more of the variation in the data than fitting environmental variables.

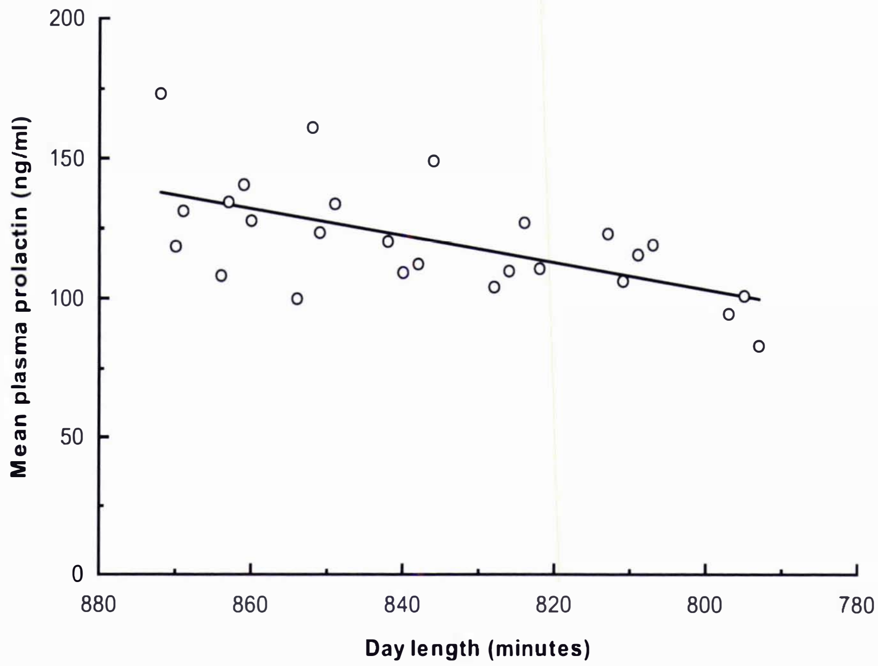


Figure 4.16 Linear regression between group means of the prolactin concentration in plasma samples taken 5 minutes after an intravenous TRH injection (0.3 μg TRH/kg lwt) and the day length in minutes on the day each group was challenged, Y = 0.4811X – 281.6, R^2 = 0.35

Thyroid stimulating hormone was undetectable in the plasma of 89% of heifers prior to TRH infusion (Figure 4.17). Maximum plasma TSH concentrations were reached between 2 and 30 minutes after TRH infusion in 95% of heifers with considerable variation between animals in the size and timing of the peak. Individual response curves were sometimes biomodal in appearance, while others had a distinct peak and then a rapid decline (Figure 4.18).

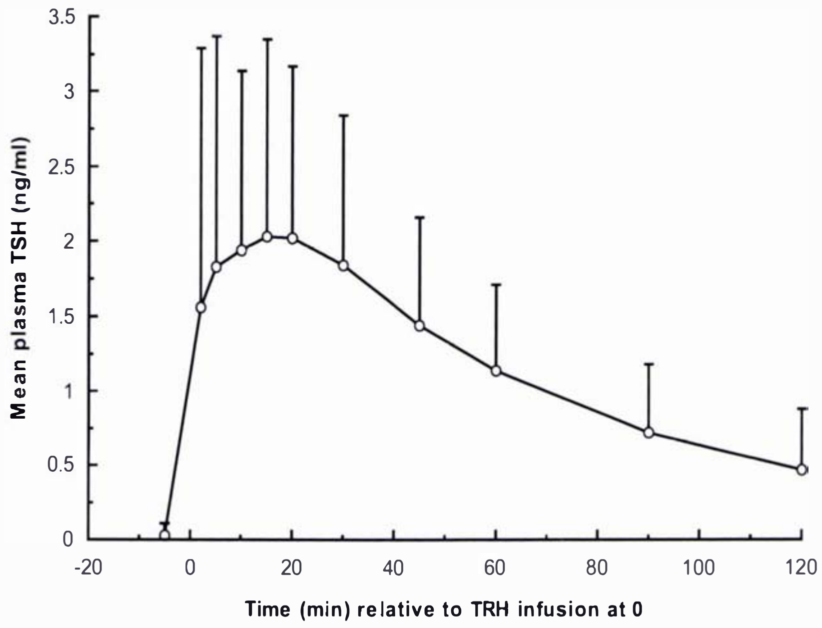


Figure 4.17 Mean TSH concentrations in the plasma of heifers, before and after an intravenous TRH injection (0.3 μg TRH/kg lwt) at time zero. Vertical bars display standard deviation.

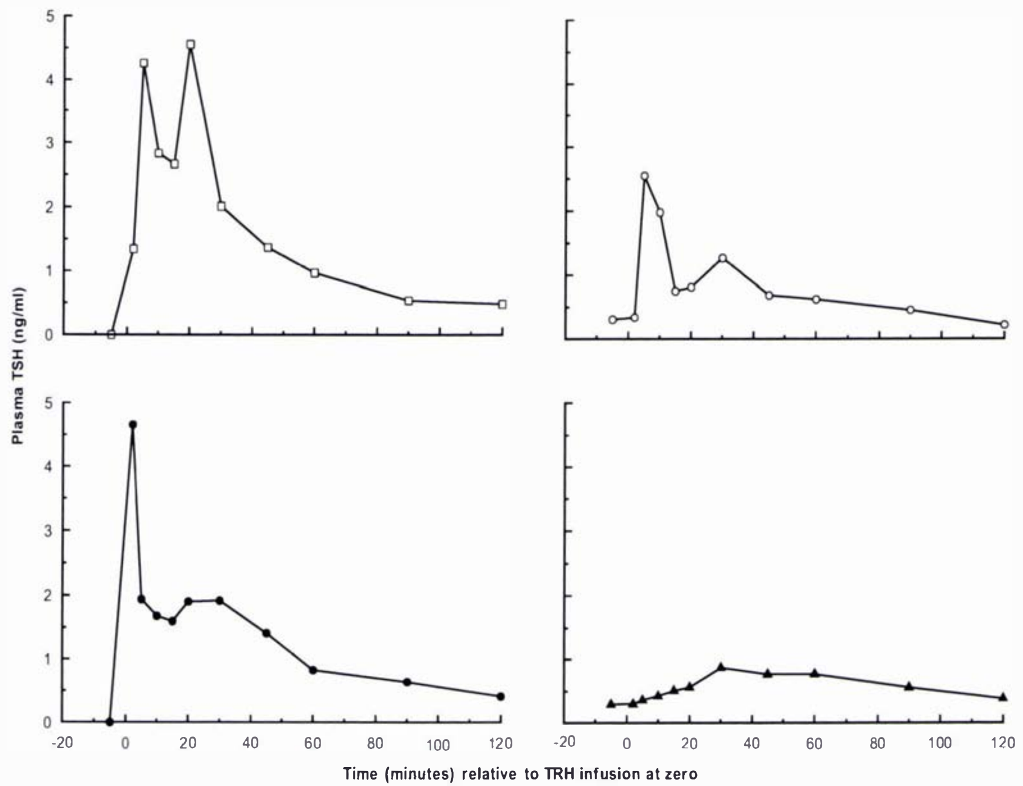


Figure 4.18 Plasma TSH concentrations of individual heifers before and after an intravenous TRH injection (0.3 μg TRH/kg lwt) at time zero. Heifer number: ○ 2051; ● 2028; □ 2174; ▲ 2037.

There was a significant effect of year ($P<0.001$) on plasma TSH concentrations at all time points. Sire did not affect TSH baseline concentrations, due to most baseline concentrations being below the detectable concentration of the assay, but did have a significant effect ($P<0.001$) on all time points measured after the TRH challenge (Figure 4.19 and Appendix D).

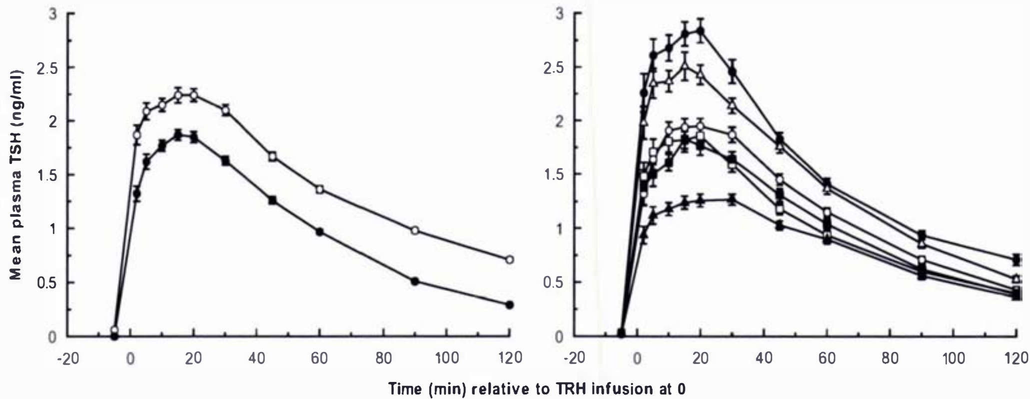


Figure 4.19 Mean plasma TSH concentrations of all heifers, grouped by challenge year (left panel; ○ 2002; ● 2003) and by sire (right panel; ○ 14415740; ● 14915827; □ 15525685; ■ 15656114; △ 16109405; ▲ 16120004), before and after an intravenous TRH injection (0.3 µg TRH/kg lwt) at time zero. Vertical bars display standard errors at each time.

Plasma NEFA concentrations prior to TRH challenge were increasing with time (Figure 4.20), and continued to increase after the TRH infusion, until maximum concentrations were reached 2-15 minutes post challenge in 84% of heifers. In general, there was a decline in plasma NEFA concentration following the maximum. Refer to Appendix D for the significance of fixed effects on plasma NEFA.

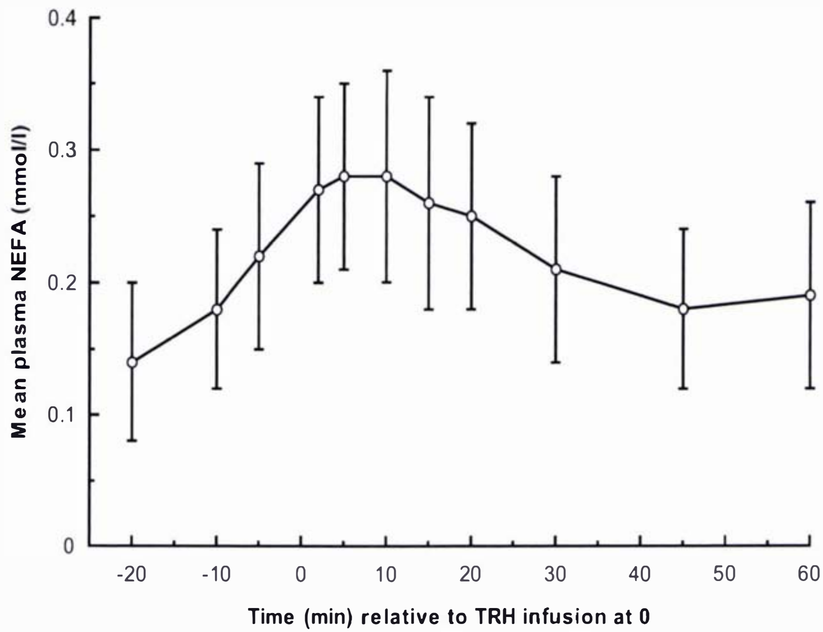


Figure 4.20 Mean plasma NEFA concentrations of heifers, before and after an intravenous TRH injection (0.3 µg TRH/kg lwt) at time zero. Vertical bars display standard deviation.

Year, group, nested within year, sire (Figure 4.21) and maternal grandsire had a significant effect of on the plasma IGF-I concentrations ($P<0.001$).

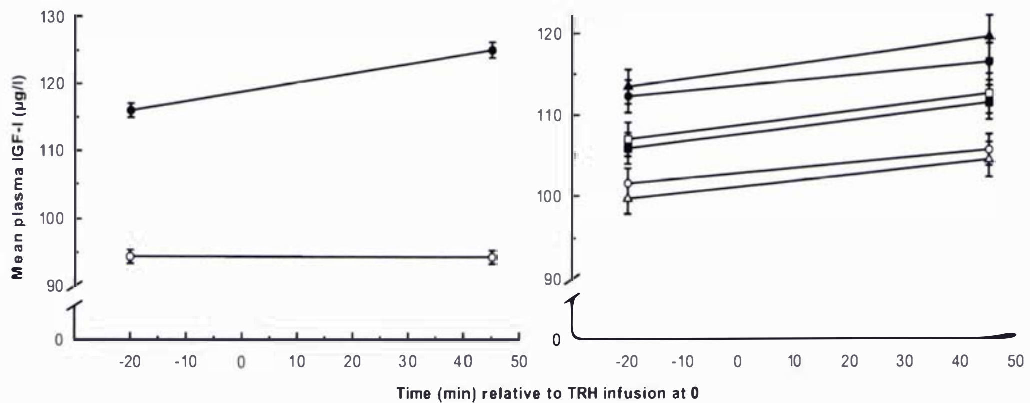


Figure 4.21 Mean plasma IGF-I concentrations of all heifers, grouped by challenge year (left panel; ○ 2002; ● 2003) and by sire (right panel; ○ 14415740; ● 14915827; □ 15525685; ■ 15656114; △ 16109405; ▲ 16120004), before and after an intravenous TRH injection (0.3 µg TRH/kg lwt) at time zero. Vertical bars display standard errors at each time.

For each variable measured in response to the TRH challenge, there was considerable variation in the calculated peak response between challenge groups (Figure 4.22). The pattern across

groups was not consistent between variables which may indicate that the response of each of these variables to the TRH challenge is influenced by other factors.

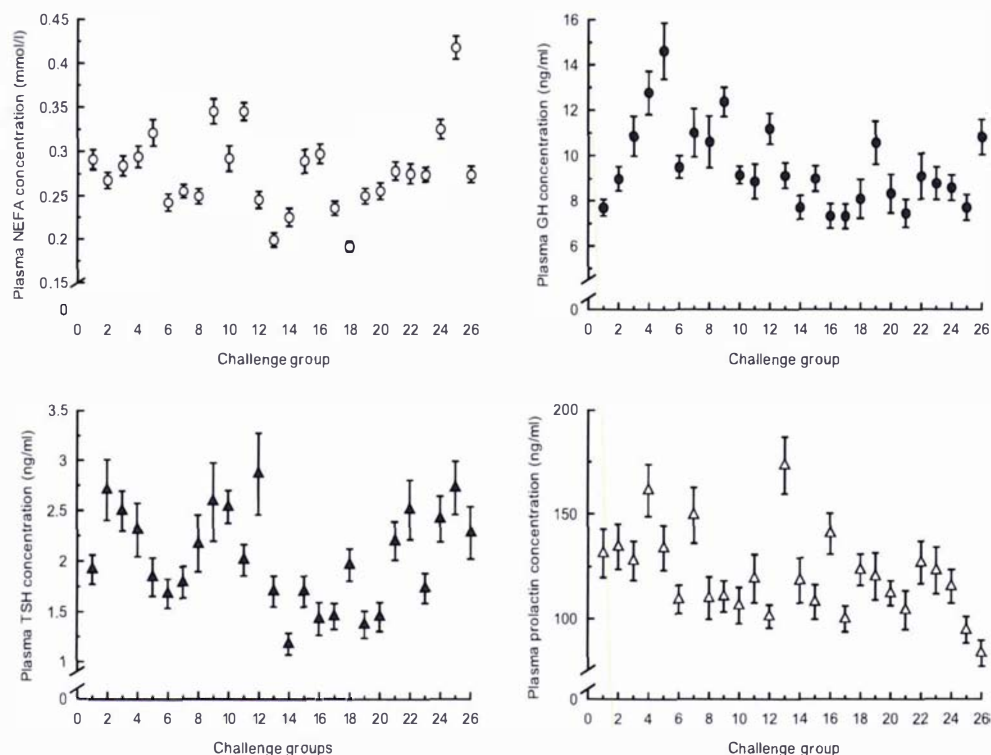


Figure 4.22 Mean plasma NEFA (○, NEFA mean for 10 minute plasma samples), GH (●, GH mean for 5 minute plasma samples), TSH (▲, TSH mean for 15 minute plasma samples), and prolactin (△, prolactin mean for 5 minute plasma samples) concentrations in response to an intravenous TRH injection at time zero (0.3 μg TRH/kg lwt), grouped by challenge group. Vertical bars display standard errors.

Discussion

There is an apparent anomaly in the 10 and 15 minute data for the 2002 GH response (Figure 4.14, left panel). Visual assessment of the plotted data suggests that the 10 and 15 minute data have been transposed. However, following investigation of these data (Chapter 3) it could not be determined if an error had occurred. Similar anomalies were not apparent in the 10 and 15 minute data of the other variables measured in response to the TRH challenge. Therefore if errors have been introduced in the 10 and 15 minute GH data these are likely to have occurred after the samples were collected and processed for storage. A biphasic plasma GH response to acute administration of GH-releasing factor has been reported in a previous study (Abribat *et al.*

1990). However it seems unlikely that a biphasic response would be present in the cohort 1 data when there is no indication of a similar biphasic response in the cohort 2 data.

The significant effects of sire on GH, TSH and IGF-I responses to TRH indicate there genetic differences in the control of secretion and possibly clearance of these hormones. Therefore, in view of the importance of these hormones, either directly or indirectly in nutrient partitioning, phenotypes calculated for these hormones may be good candidates for finding QTL that are correlated with milk or milk solids production.

4.6 Finalised phenotypes

Following initial assessment of the response curves and adjustment of the data for fixed and random effects the metabolic phenotypes were then calculated. Selection of appropriate response variables is vital for the detection of genetic differences and therefore QTLs. Phenotypic variables were decided upon at this point in the study, following assessment of this data set due to it being the definitive data set for 18 month old, early gestation dairy heifers combined with an extensive search of the literature to obtain useful variables that have been reported. The data from the current study will provide a much better understanding of the average response variables to these challenges due to the number of animals in the study and their age and physiological state.

Adrenaline challenge phenotypes

The maximum and peak phenotypes for glycerol and NEFA were used as indicators of the maximum mobilisation of triacylglycerol from adipose tissue. It was hoped time to maximum would give an indication of the sensitivity or responsiveness of adipose tissue to adrenaline. The slope of the decay curves were calculated as an indicator of tissue uptake of the metabolite from the circulation. Area under the curve (AUC) was calculated to quantify the response NEFA, glycerol or glucose to the adrenaline challenge and takes into account both release and uptake.

Glucose challenge phenotypes

Several variables were calculated to give an indication of the slope of the glucose decay curve, these were *k* (fractional turnover rate (%/min)), T½ (glucose clearance half times), rate of decrease calculated as the slope of the glucose response curve after the peak. Slope of the glucose decay curve reflects the insulin concentration and the sensitivity of the tissues to insulin as well as sensitivity of the tissues to glucose in terms of non insulin mediated glucose uptake. Glucose concentrations declined more rapidly in high breeding index compared with low breeding index 8 month old bull calves as well as lactating heifers and multiparous cows after a glucose infusion (Mackenzie *et al.* 1988, Michel *et al.* 1991).

There is likely to be a genetic component to control the sensitivity of the pancreas to glucose, therefore, insulin variables that give an indication of this would be useful. The slope of the insulin response curve from baseline to peak, the AUC up to 15 minutes post-glucose infusion, peak, insulin maximum and time to insulin maximum (definitions Appendix E, Table 9.4) were the phenotypes calculated to give an indication of sensitivity of the pancreas to glucose. Insulin concentrations were significantly greater in heifers and bulls with a high breeding index for milk fat yield than those with a low breeding index in response to exogenous glucose (Mackenzie *et al.* 1988, Michel *et al.* 1991, Xing *et al.* 1993), indicating that insulin response to exogenous glucose has a genetic level of control.

Insulin clearance was determined using two methods to calculate the slope of the response curve after the peak. If insulin is cleared from the circulation quickly there will be less time for it to cause glucose uptake compared with an animal where the insulin is cleared more slowly.

Minimum NEFA concentration, time to minimum NEFA concentration and the slope of the disappearance of NEFA were calculated as variables to give an indication of the effect of insulin on adipose tissue.

TRH challenge phenotypes

The trapezoidal method, which involved summing the areas of adjacent sample times, was used for calculating area under the curve, which means that the possible reversal of the 10 and 15 minute sample concentrations will not change the calculated values. Similarly, the peak in the response curve was the average of the 5, 10 and 15 minutes samples and would be unaffected by transposing the values for the 10 and 15 minute samples. However, the rate of clearance of the GH would be affected by the 10 and 15 minute samples being transposed. Consequently, the phenotypes used for QTL analyses should not be affected by this possible error, except the GH rate of clearance phenotype.

Summary

After reviewing the endocrine challenge literature and integrating the challenge data, the main phenotypes chosen were:

- baseline, mean of the pre-challenge samples (Fröhli and Blum 1988, Grochowska *et al.* 2001, Løvendahl *et al.* 1994, Père *et al.* 2000, Shingu *et al.* 2002)
- peak, mean of the two time points at which the majority of animals reached maximum concentration (Baumgard *et al.* 2002, Lemosquet *et al.* 1997, Sørensen *et al.* 2002) or the concentration at the sample time which the most animals reached maximum (Grochowska *et al.* 2001)
- maximum, the greatest plasma concentration of the hormone or metabolite after the challenge (Baumgard *et al.* 2002, Lapierre *et al.* 1990, Perriello *et al.* 2005, Radcliff *et al.* 2003)
- time to maximum (Baumgard *et al.* 2002, Perriello *et al.* 2005)
- AUC, calculated using the trapezoidal method after exclusion of baseline (Bunting *et al.* 1994, Lacasse *et al.* 1991, Lapierre *et al.* 1990, Lemosquet *et al.* 1997, Radcliff *et al.*

2003) correction for baseline allowed calculation of AUC without the confounding effects of variation in pre-challenge values (Kolver *et al.* 2001)

- slope of the decay part of the curve, calculated using several different methods (Appendix E) (Itoh *et al.* 1998, Kaneko 1997, Løvendahl *et al.* 1994, Wilmink 1987).

A list of the phenotypes generated and calculation methods used are in Appendix E. These phenotypes were calculated to provide informative indicators of metabolic control points that are important to milk production. Thus, these phenotypes provide the best data for finding metabolic QTL that are co-located with milk production QTL. The method used to conduct QTL searches with the metabolic phenotypes and the results are described in Chapter 5.

CHAPTER 5

QTL mapping and selection

5.1 Introduction

The purpose of this chapter is to outline the data manipulation and processing required to generate a list of candidate genes. The candidate genes were then assessed for their relevance to the QTL in the region of interest. Discussion of the genes will occur in the next chapter. The key steps in the process were:

1. generation of phenotypes from adjusted phenotypic data
2. run the data through the QTL mapping interface
3. generate output for the significant QTL
4. assess the output and decide which chromosomal regions are of interest and warrant further investigation
5. generate a list of candidate genes for the region of interest
6. investigate the biochemical function of these candidate genes to try and establish which are the most likely to be causing the QTL seen that region.

Each of these steps will be discussed in this chapter.

5.2 Phenotype generation

An overview of the phenotypes calculated using SAS (version 8.2, 1999-2001 by SAS Institute Inc., Cary, NC, USA) was given in Chapter 4. In total 107 (21 glucose challenge; 23 adrenaline challenge; 51 TRH challenge) phenotypes were calculated for each heifer that underwent the experimental challenges. Once these phenotypes had been generated, the data were transferred into tables, one per challenge. These files were then used to run the preliminary 'multimasher' analyses.

5.3 QTL analysis using WEBQTLANA

WEBQTLANA is a tool developed by scientists at Livestock Improvement Corporation (LIC), which enabled web-based QTL analysis to be performed on the FJXB experiment data. This web-based tool was developed by Dr Richard Spelman in Fortran 90 for mapping QTL and uses a suite of Haley Knott regression programs based on least squares regression methodology (Knott *et al.* 1996). Three QTL models were fitted: additive, additive + dominance in a line of descent setting and a half sib model. For each putative QTL position, linear least squares can be used to regress the trait value for each animal onto their additive (a) and dominance (d) coefficients (Haley *et al.* 1994). The model gives estimates for a and d for that position. The procedure is repeated for chosen fixed positions (every cM) through a linkage group and the best estimate of the QTL effects and position are given where the residual sum of squares is minimised (Haley *et al.* 1994). For a fixed position of QTL, the ratio of the regression mean square to the residual mean square provides the variance (F) ratio test statistic (Haley *et al.* 1994).

Initially the phenotypes were run through a 'multimasher' scan in WEBQTLANA, this involved a genome wide scan for each phenotype. The 'multimasher' scan was run at 500 permutations to estimate critical values. It gave an output that contained the phenotype, the chromosome and p-values for each of the three QTL models fitted (Figure 5.1). This output was used to identify which chromosomes had potentially significant QTL for each phenotype and therefore required further investigation.

Following identification of the potentially significant QTL from the 'multimasher' scans, an individual file for each of the phenotypes with the corresponding animals key (an identification number that is unique for each animal in the Friesian-Jersey crossbred trial database) were generated. These phenotype files were used to run further QTL analyses on the chromosomes that had been identified by the 'multimasher' scans. These analyses were of a greater accuracy than the initial 'multimasher' scan due to the greater number of permutation tests (the number of repetitions of the analysis) and bootstrapping (used to determine a confidence interval for the

location of the QTL), which were 10,000 and 1000, respectively. The permutation tests determine an experiment-wide significance level for the QTL by re-computing the test statistic for each new sample, generating an empirical distribution from the null hypothesis. The null hypothesis was that there was no phenotype-genotype associations. Datasets for the null hypothesis were generated by using random shuffling to assign genotypes to the phenotypic data for the permutations tests and the analysis was re-run 10,000 times (Spelman *et al.* 1996).

MultiMasher: The results from a scan.

Phenotype	Chromosome	L-o-D a P-value %	L-o-D a+d P-value %	half-sib P-value %
GH_auc_total	1	-1	-1	13
GH_auc_total	2	-1	17.2	1.4
GH_auc_total	3	-1	-1	84.2
GH_auc_total	4	-1	-1	23.8
GH_auc_total	5	15	22.2	87
GH_auc_total	6	-1	-1	23
GH_auc_total	7	-1	-1	76.8
GH_auc_total	8	11.6	21.2	70.2
GH_auc_total	9	5.2	16.6	3.8
GH_auc_total	10	-1	0.8	68.8
GH_auc_total	11	-1	23.2	8.6
GH_auc_total	12	-1	15.8	13.8
GH_auc_total	13	3.6	5.2	0.8
GH_auc_total	14	-1	-1	27

Figure 5.1 Example of the output generated by QTLANA from a 'multimasher' scan. The output shows the phenotype used in the analyses, the chromosomes scanned and the P-value expressed as a percent for the three QTL models fitted; additive (a), additive + dominance (a+d) in a line of descent (L-o-D) setting and half sib model. The highlighted values are those that are significant at the 5% level. The -1 *P*-values occur where the F-values were too low for P-values to be estimated via permutation, this saved computational time.

QTL mapping was conducted for each phenotype on each chromosome that the multimasher scan found a significance level of 5% or less. This resulted in more than 1,300 separate QTL analyses being run in WEBQTLANA. Only those QTL that were significant at the 1% level were included in the next stage of the analyses (Appendix F). An example of the output generated for a single chromosome, BTA 14, and phenotype, IGF-I mean, is shown in the subsequent section. The text in italics provides an explanation of each part of the output.

Example output from WEBQTLANA

QTL Analysis File preparation
Phenotyped Animals in uploaded file: 882 Genotyped Animals for chromosome: 864
Genotyped Animals not phenotyped: 12 Genotyped Animals all unknown markers: 16
Genotyped Animals and Phenotypes: 836
Animals used in Analysis: F0SS 6; F0DS 6; F0SD 59; F0DD 755; F1S 6; F1D 771; F2 836

Analysis Results

********** ADDITIVE AND DOMINANCE QTL MODEL ******
Trait: 1

Best QTL:

F Ratio 7.73 *(Highest F-value on the chromosome for the additive and dominance model)*

Location 47 *(Location of the highest F-value on the chromosome for this model)*

segregation distortion (Dis & Het) 1.031 -0.494

Segregation distortion is calculated as a deviation of the observed average additive, dominance and parent-of-origin coefficients to their expectations under the null hypothesis of Mendelian segregation. A statistical test is performed by computing the difference between observed and expected coefficients and the estimated standard error of that difference. Under the null hypothesis of no segregation distortion, this ratio is distributed as a t-distribution, and therefore the graph with segregation distortion contains horizontal lines at t=-2.0 and t=+2, corresponding to nominal P-values of approximately 0.05 (two-sided).

additive effect	se	dominance effect	se
-3.405	0.868	0.134	1.277

(The additive effect and its standard error and the dominance effect and its standard error)

************* ADDITIVE QTL MODEL ***************
Trait: 1

Best QTL:

F Ratio 7.72 *(Highest F-value on the chromosome for the additive model)*

Location 47 *(Location of the highest F-value on the chromosome for this model)*

segregation distortion (Dis) 1.031 *(as above)*

additive effect	se	dominance effect
-3.408	0.867	0.000

(The additive effect and its standard error)

ADDITIVE AND DOMINANCE P-VALUES FROM PERMUTATION TESTING
Results from 10000 permutations for Interval mapping

95% threshold	99% threshold	99.9% threshold	My F-Value	P-Value(%)
5.06685	6.75104	9.32670	7.72609	0.43

(Critical values at the different threshold levels. P-value calculated for the F-value from the analysis)

ADDITIVE P-VALUES FROM PERMUTATION TESTING
Results from 10000 permutations for Interval mapping

95% threshold	99% threshold	99.9% threshold	My F-Value	P-Value(%)
3.48651	5.11025	7.28430	7.72051	0.06

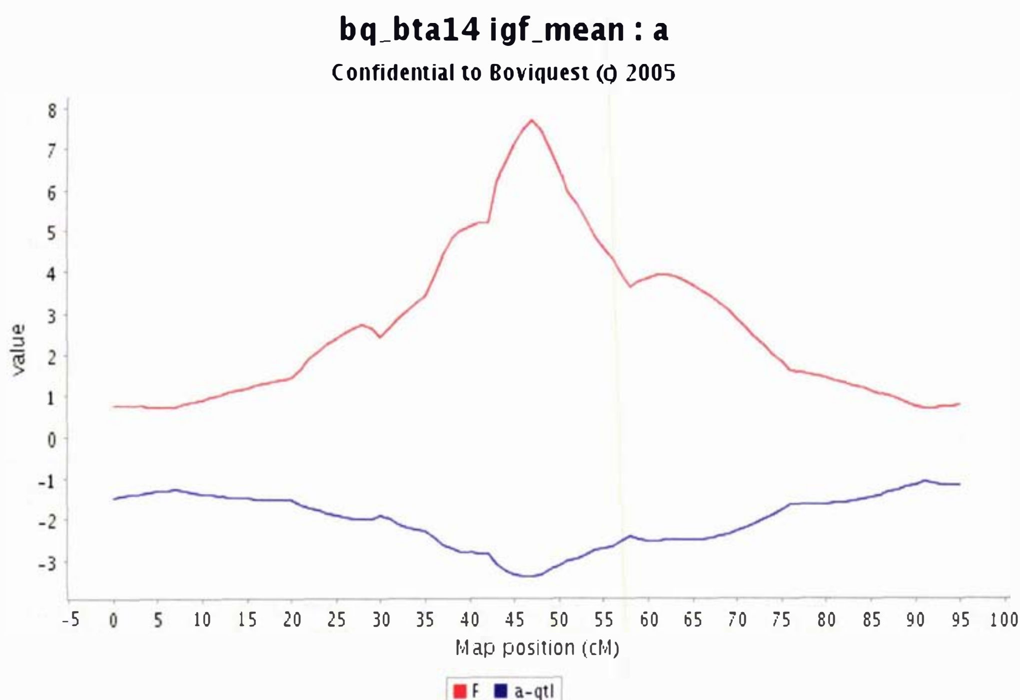
(Critical values at the different threshold levels. P-value calculated for the F-value from the analysis)

95% CI for QTL location
38.00 cM - 65.00 cM *(Confidence interval from the bootstrapping)*

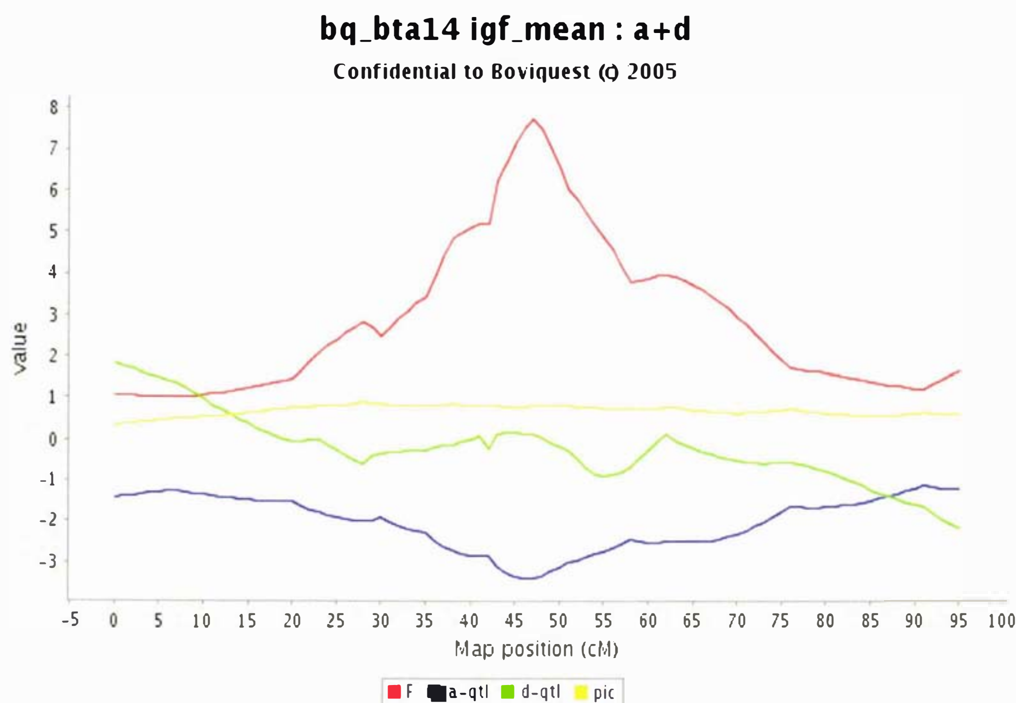
Additive+Dominance Model
Maximum F Score 7.73
Location 47.0
P Value (%) 0.43

Additive Model
Maximum F Score 7.72
Location 47.0
P Value (%) 0.06

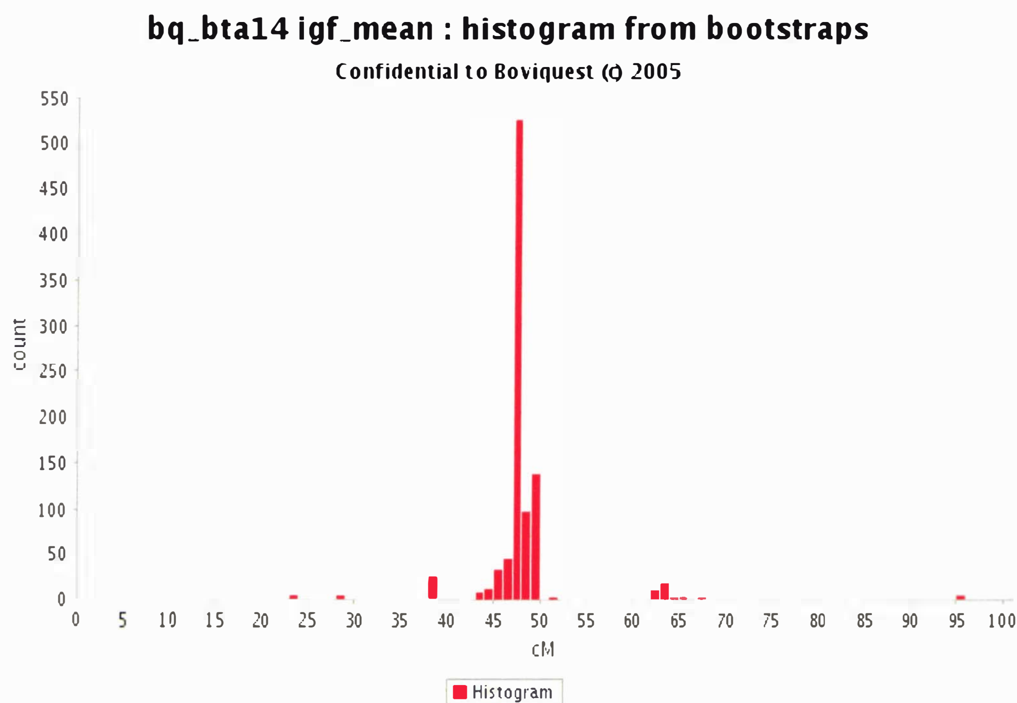
(Summary statistics from above tables for additive+dominance and additive models)



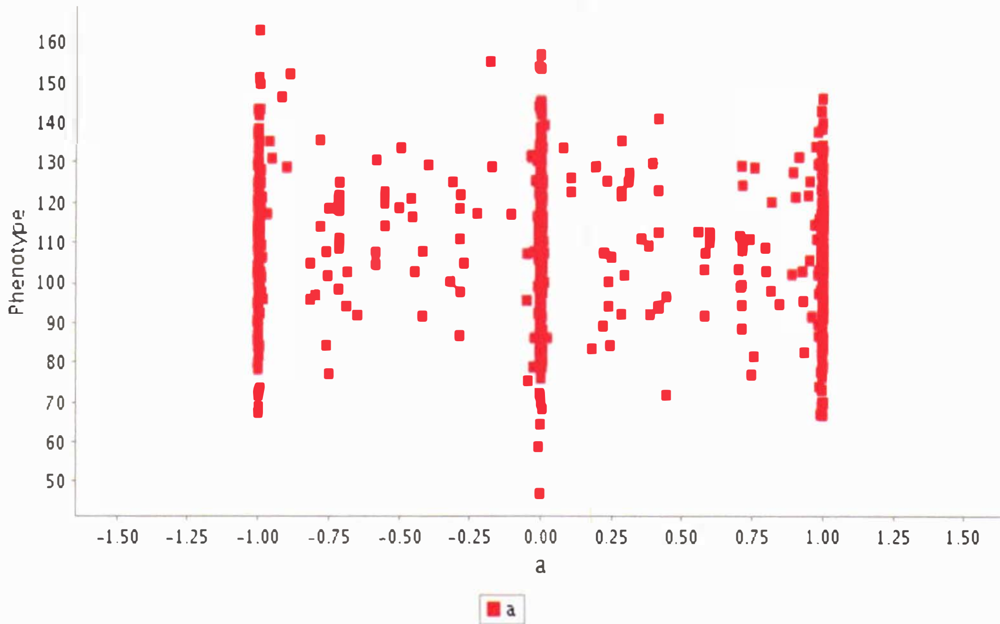
The F-value test statistic and the additive model QTL have been plotted against chromosome position. The maximum point of the F-value curve indicates the most likely position of the QTL.



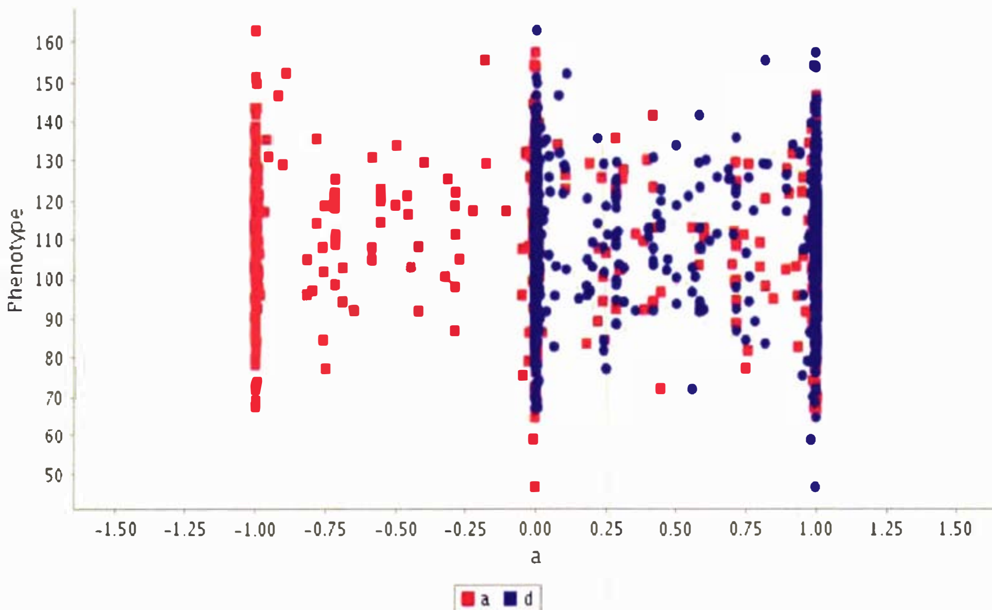
The F-value test statistic, additive and dominance model QTL and pic (see below) have been plotted against chromosome position. The maximum point of the F-value curve indicates the most likely position of the QTL. Polymorphic information content (pic) is a measure of how informative the sires are and is calculated as the ratio of the variance in conditional probability at any position, to the variance when true descent is known. Generally the true descent is not known and must be inferred from informative flanking markers.



Bootstrapping is the generation of a confidence interval for the position of the QTL peak created by sampling with replacement individuals from the data-set for that sire. The number sampled was equal to the number of individuals in the original dataset for that sire. Bootstrapping was repeated 1000 times and the peak calculated for each repetition was plotted in a histogram.



A scatter plot of the phenotype values with the respective regression coefficients for additive model. Each point on the scatter plot is an animal. The data points at -1 are the animals that have received 2 Jersey chromosomal sections, those at 0 have 1 Friesian and 1 Jersey chromosomal sections and those at the 1 have 2 Friesian chromosomal sections.



In this scatter plot the red data are exactly the same as in the previous scatter plot and are for the additive model as already mentioned. The blue dots are for the dominance coefficients. All the d's at zero are the animals that have either received 2 Jersey chromosomal sections or two Friesian chromosomal sections, i.e. -1 or 1 for the additive scatter plot and therefore are 0 for dominance scatter plot.

5.4 Endocrine and milk production QTL

A list of QTL, significant at the 1% level, for milk production and metabolic phenotypes was generated (Appendix F). The milk production QTL were generated using the same method as that discussed for the metabolic QTL. This enabled the identification of chromosomal regions with metabolic QTL in close proximity to milk production QTL. There were many chromosomal regions that contained either metabolic or milk production QTL (Appendix F and Table 5.1). There were very few chromosomal regions in which metabolic and milk production QTL were co-located. A chromosomal region in which metabolic and milk production QTL occurred in close proximity was selected because the causes of a genetic correlation are either pleiotropy or genetic linkage. Pleiotropy is the multiple effects of one gene, that is, one gene affects the genetic expression of two or more traits. Genetic linkage occurs when two genes affecting separate traits are situated close together on the same chromosome, preventing the genes from segregating independently at meiosis (Falconer and Mackay 1996).

Table 5.1 Examples of regions of interest found using the metabolic and milk production QTL

BTA	QTL	cM	P-values	Description of region
2	NEFA decay, prolactin peak, days in milk	131	0.0003 – 0.0047	End of chromosome
9	Insulin – clearance, peak, total area. GH – peak, AUC until peak	31-38	0.0001 – 0.0088	A large number of insulin QTL over a relatively large gene region
14	IGF-I; day 60 yields – milk, lactose, crude protein, casein, whey protein, total protein; glycerol clearance	47-51	0 – 0.0031	Highly significant endocrine and milk QTL in this region
18	Insulin phenotypes	21	0.0018 – 0.0098	A large number of insulin QTL all at the same location on this chromosome
27	NEFA base, milk fat % and TSH clearance	42-49	0.0002 – 0.0096	Both the endocrine and milk traits were significant

Some regions were not selected for investigation, even though they contained a large number of QTL, because the associations between the QTL and candidate genes have already been

established. For example, at 0 CM on BTA 14 in the current study there were QTL for milk fat percentage at all stages of lactation. This region was not considered further because the QTL and the gene most likely responsible, DGAT1, have already been reported (Grisart *et al.* 2002). Even though these regions were not investigated further in this study, confidence in the techniques used to identify QTL and their relationships with phenotypes is warranted given that similar regions have been identified in other studies.

The chromosomal regions to be considered for candidate gene studies were selected on the basis of low P-values, high F-values, a well-defined location and a high density of QTL of similar phenotypes. The regions that contained either milk production or metabolic QTL, were excluded as the purpose of the study was to find candidate genes that affect both metabolic and milk production phenotypes significantly. The region selected for a candidate gene study was BTA 14 due to the region containing both endocrine and milk production QTL, the low P-values and high F-values of the QTL.

A primary objective of this study was to identify chromosomal regions in which metabolic and milk production QTL were co-located. It was hoped that these regions would contain genes that had a significant impact on the control of milk production. However, one of the major findings from this study was that there are only a very small number of regions that contained both metabolic and milk production QTL located within a few cM of each other. There were a large number of chromosomal regions where clusters of similar metabolic or milk production QTL were co-located but it was rare for metabolic and milk production QTL to be co-located (Appendix F). Perhaps this is not a surprising finding, given the difficulty in establishing consistent and repeatable physiological indicators of milk production demonstrated in previous studies, reviewed by Woolliams and Løvendahl (1991). On a number of chromosomes (BTA 1, 5, 6, 8, 11, 14, 25 and 27), clusters of metabolic QTL were co-located and milk production QTL were co-located on distinct regions of the same chromosome. It may be that genes underlying the clusters of metabolic and interact with genes underlying milk production QTL due to chromosome folding. Thus, expression of genes influencing milk production traits may be

activated by genes controlling metabolic traits at distant locations on the same chromosome (Kleinjan and van Heyningen 2005).

Chromatin is a highly organised and dynamic nucleoprotein structure that plays a pivotal role in gene regulation by modulating access to DNA during gene replication and transcription (Joyce 2007, O'Reilly and Greaves 2007). Changes in chromatin architecture and formation of chromatin loops have been proposed as a mechanism for bringing together DNA and proteins within a defined sub-region of the nucleus to facilitate activation or silencing of gene expression (O'Reilly and Greaves 2007). The genomic regions containing regulatory elements can stretch as much as 1 megabase in either direction from the transcription unit. Some or all of these regulatory elements can be situated within the introns of neighbouring gene(s), often with a function that is unrelated to the regulated gene (Kleinjan and van Heyningen 2005). The regulatory domain is first activated by transcription factor binding and chromatin modifications at the remote upstream elements. These changes then extend along the chromatin domain from the upstream elements to the promoter, producing a fully poised state at each of the conserved elements (enhancers and promoters) throughout the regulatory domain (Vernimmen *et al.* 2007). Remote regulatory elements appear to play an important role within the stepwise orchestration of promoter assembly. They bring about a physical interaction between promoters and the pre-initiation complex (a complex of general transcription factors and RNA polymerase II) leading to chromatin remodelling and transcriptional initiation (Vernimmen *et al.* 2007). Thus, it would seem that there is long-range regulation of gene expression. This has important implications for conducting QTL searches. A QTL location may be identified at the site of chromosomal rearrangement, that apparently affects one gene, however, the rearrangement may dissociate remote control elements from a more meaningful candidate gene located some distance away (Kleinjan and van Heyningen 2005). The long-range regulation of gene expression will need to be considered in future studies. In the current study only milk production and metabolic QTL that were in close proximity were considered. This imposed a reasonable limit on the number of

possible QTL combinations requiring consideration and consequently also placed a limit on the potential candidate genes that required investigation.

5.5 Candidate genes

Once the chromosomal region to be studied was selected, there were a number of steps taken to generate the list of candidate genes. Followed by assessment of which genes were most likely to be responsible for the QTL in the region of interest. The steps were:

1. Obtain the estimate of the best location for the QTL from the WEBQTLANA output.

2. Look up the gene markers that were situated close to this location in the FJXB gene maker file. One nearest to the QTL location and two that were approximately 10cM either side of the QTL location.

3. Comparative livestock annotation system (CLAS) was used to generate the list of candidate genes. CLAS is an LIC inhouse (developed by Alexander Kvasz and Wouter Coppieters, Molecular Genetics, Faculty of Veterinary Medicine, University of Liège, Belgium) bioinformatics platform that allows the bovine and human genomes to be aligned. Thus, it utilises gene annotation that has occurred for the human genome. Firstly, the chromosome of interest and the gene maps to be used were selected then the comparative view window was opened. The gene maps used were from the USDA (United States Department of Agriculture) and SIAG (Shirakawa Institute of Animal Genetics) along with the gene marker map for the Friesian-Jersey crossbred trial. At the time these steps were conducted for the current study the bovine map had not been annotated so comparative mapping was used to generate the list of candidate genes.

4. Once the comparative view window was open then the gene markers near the QTL of interest, obtained in step 2, were searched for and identified on the three gene maps (USDA, SIAG and FJXB). The markers were used to define the region from which the candidate gene list was generated.

5. A report was generated for the selected region. The report contained the following, for each gene, where known:

   - HGNC (HUGO (The Human Genome Organisation) Gene Nomenclature Committee) symbols

   - Ensembl gene identification numbers. Ensembl is a joint project between EMBL - European Bioinformatics Institute (EBI) and the Wellcome Trust Sanger Institute (WTSI) to develop a software system that produces and maintains automatic annotation on selected eukaryotic genomes.

   - A description of the gene function

   - A RefSeq or Swiss-Prot/TrEMBL accession number. The Reference Sequence (RefSeq) is the public database of nucleotide and protein sequences built by the National Centre for Biotechnology Information (NCBI) (Pruitt *et al.* 2005). Swiss-Prot/TrEMBL accession numbers were obtained from the Universal Protein Resource (UniProt) database. UniProt is a centralised resource for protein sequences and functional information, formed in 2002 through the amalgamation of the Swiss-Prot + TrEMBL (created by the Swiss Institute of Bioinformatics and European Bioinformatics Institute) and PIR-PSD (created by the Protein Information Resource group at Georgetown University Medical Center and National Biomedical Research Foundation) protein databases (Bairoch *et al.* 2005).

6. The Ensembl gene identification numbers were extracted from the report.

7. The list of Ensembl numbers was entered into a gene identification (ID) code converter website located at http://idconverter.bioinfo.cipf.es/ (15 March 2007). The website was used to convert the Ensembl numbers to Locuslink ID (NCBI). LocusLink was developed by NCBI as a cross-referencing tool that anchors an official gene name, gene

aliases, database IDs, phenotypes, map positions, sequence accession numbers, and other identifiers to a stable LocusLink ID number (Pruitt and Maglott 2001).

8. An initial investigation of each gene was conducted. Gene networks and gene function information were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, http://www.ingenuity.com/, (5 April 2007)) and GeneCards® (Rebhan *et al.* 1997).

9. Once the initial investigation of each gene in the candidate list was completed, the information was assessed. Genes that did not have a function that could logically be related to the QTL and genes for which there was no information were discarded. The genes that had functions that could potentially be related to the QTL of interest were then investigated in published literature for their relevance the milk and metabolic phenotypes in the region of interest. One of the major difficulties with this work is the numerous synonyms or aliases for each gene. This meant that literature searches had to encompass as many synonyms as possible. If not all synonyms were known this would have reduced the effectiveness of the literature searches.

5.6 Summary

In total 107 (21 glucose challenge; 23 adrenaline challenge; 51 TRH challenge) phenotypes were calculated on adjusted data for each heifer that underwent the experimental challenges (Chapter 4). These data were then subjected to preliminary analyses in WEBQTLANA that involved a genome wide scan of each phenotype. The output was used to identify the chromosomes that had potentially significant QTL at a significance level of 5% or less. More than 1,300 separate analyses were then run through WEBQTLANA again using parameters that conferred greater accuracy to the analyses. A list of QTL, significant at the 1% level, for milk production and endocrine phenotypes was generated (Appendix F).

The processes described in this chapter led to a large number of QTL being mapped using the metabolic and milk production phenotypes. 581 QTL were significant at the 1% level and 275

of them were QTL of endocrine phenotypes (Appendix F). The large number of QTL suggests that the metabolic challenges did perturb the physiology of the animals in such a way that it allowed likely genetic control mechanisms to be identified. Certain phenotypes were strongly associated with particular chromosomes, for example QTL for insulin phenotypes were found on chromosomes 9, 15, 18 and 25, and QTL for TSH were found on chromosomes 3, 11 and 13 (Appendix F).

A major finding of this study was the very small number of regions in which metabolic and milk production QTL were co-located. However, it is possible that gene regulatory elements are remotely located, as much as 1 megabase, in either direction from the transcription unit. Some or all of these elements may be situated within the introns of neighbouring gene(s), often with a function that is unrelated to the regulated gene (Kleinjan and van Heyningen 2005). Long-range regulation of gene expression adds another level of complexity to conducting QTL searches (Kleinjan and van Heyningen 2005).

The region selected for a candidate gene study was 47-51 cM of BTA 14 due to the region containing both endocrine and milk production QTL, the low P-values and high F-values of the QTL. The candidate gene list was generated using a comparative gene map annotation programme. An initial investigation of each gene and its function was carried out using two websites that mine data from a number of literature sources. Assessment of this information was conducted and a number of genes were discarded from the study. The genes that had functions that could potentially be related to the QTL of interest were then further investigated using published literature. Investigation of the selected genes is described in the Chapter 6.

CHAPTER 6

Candidate genes in region of interest on chromosome 14

6.1 Introduction

In previous chapters, the metabolic challenges, generation of phenotypes, QTL analyses and generation of a list of genes in the chromosomal region of interest have been discussed. The objectives of this chapter are to investigate the function of each of the genes in the list and draw conclusions as to which of these genes would be the most likely to act as genetic control mechanisms of the metabolic and milk production QTL.

A region from 47 to 51cM on *Bos Taurus* autosome 14 (BTA14) was selected as the region of interest due to the proximity of metabolic and milk production QTL and the high F-values and low P-values (Chapter 5). This region includes QTL for baseline insulin-like growth factor-I (IGF-I), 60 day yields for milk, lactose, crude protein, total protein, casein and whey protein (Table 6.1).

Table 6.1 The predicted locations (cM) for the QTL for mean concentrations of baseline insulin-like growth factor 1 (igf_mean), the first 60 days of lactation (d60) milk yield (pmy), lactose yield (ply), crude protein yield (pcpy), total protein yield (ptpy), casein yield (pcy) and whey protein yield (pwpy) as determined by the additive (A) and additive + dominance (A+D) QTL models together with the level of significance (P-value) the maximum F-value and the 95% confidence interval (95% CI) of the QTL location (cM).

Analysis	Phenotype	Predicted location (cM)	P-value	Maximum F-value	95% CI
A	igf_mean	47	0.0006	7.72	38 - 65
A+D	igf_mean	47	0.0043	7.73	38 - 65
A+D	d60_pmy	48	< 0.0001	11.07	13 - 56
A+D	d60_ply	48	0.0001	11.18	21 - 56
A	d60_ply	49	< 0.0001	10.37	21 - 56
A	d60_pmy	49	0.0001	10.09	13 - 56
A+D	d60_pcpy	51	< 0.0001	11.72	37 - 55
A	d60_pcpy	51	< 0.0001	11.31	37 - 55
A	d60_ptpy	51	< 0.0001	11.26	38 - 54
A	d60_pcy	51	0.0001	11.14	35 - 56
A+D	d60_ptpy	51	0.0001	11.59	38 - 54
A+D	d60_pcy	51	0.0002	11.57	35 - 56
A	d60_pmsy	51	0.0002	8.72	22 - 60
A	d60_pwpy	51	0.0012	7.92	20 - 54

Table 6.1 Continued.

Analysis	Phenotype	Predicted location (cM)	P-value	Maximum F-value	95% CI
A+D	d60_pmsy	51	0.0016	9.53	22 - 60
A+D	d60_pwpy	51	0.0031	7.92	20 - 54

A list of 105 genes (Appendix G) contained in this region was generated according to the steps described in Chapter 5. There were 28 genes in this list that did not have a name or a description of their function and were eliminated from the study. The remaining genes were investigated for any apparent relevance to the QTL in the region.

Initially information about each gene was obtained using Ingenuity Pathways Analysis (Ingenuity® Systems, http://www.ingenuity.com/ 30/06/07) and GeneCards® (Rebhan *et al.* 1997) last accessed 15/12/07. These two websites both provided summaries of the currently available information about each gene. These summaries were used to identify the genes that might be relevant to the QTL of interest in this investigation. Thirty-seven genes were selected for a more detailed investigation of their function through a search of the literature (Table 6.2).

The literature search was facilitated by the use of GeneCards® ((Rebhan *et al.* 1997) last accessed 15/12/07). This website contains a section that displays synonyms and aliases for the relevant gene, as extracted from GDB (GDB Human Genome Database), OMIM (Online Mendelian Inheritance in Man), HGNC, Entrez Gene (NCBI's database for gene-specific information), UniProt (Swiss-Prot/TrEMBL), and Ensembl. This tool was exceptionally valuable, as each gene had numerous aliases and synonyms and knowing these enabled a more thorough search of the literature.

An extensive literature search (more than 300 journal articles) was completed to identify the most likely candidate genes. Genes that were found not to have any apparent relevance to lactation or nutrient partitioning were eliminated. Other genes fell into the category of possibly important, but were not presented after critical assessment of the literature and relevance of the genes to the QTL in question. A small number of genes that appeared to be highly relevant to

the QTL in the region were chosen for discussion. Every effort was made to use the most recently available information about the genes under investigation. However, new information about genes and gene function becomes available daily in gene databases or in journal publications. It is very likely that this new information will identify additional genes that will be candidates for the QTL in this study.

Table 6.2 Genes that were investigated for relevance to QTL of interest between 47 and 51 cM on BTA14

Symbol	Ensembl gene	Name/Description
RB1CC1	ENSG00000023287	RB1-inducible coiled-coil 1
OPRK1	ENSG00000082556	Opioid receptor, kappa 1
NPBWR1	ENSG00000183729	Neuropeptides B/W receptor type 1
ATP6V1H	ENSG00000047249	ATPase, H^+ transporting, lysosomal 50/57kDa, V1 subunit H
TTPA	ENSG00000137561	tocopherol (alpha) transfer protein (ataxia (Friedreich-like) with vitamin E deficiency)
SDCBP	ENSG00000137575	Syndecan binding protein (syntenin)
RRS1	ENSG00000179041	Ribosome biogenesis regulatory protein homolog
RPS20	ENSG00000008988	ribosomal protein S20
TRIM55	ENSG00000147573	tripartite motif-containing 55
RGS20	ENSG00000147509	regulator of G-protein signalling 20
RAB2	ENSG00000104388	RAB2, member RAS oncogene family
PENK	ENSG00000181195	proenkephalin
PDE7A	ENSG00000205268	phosphodiesterase 7A
TGS1	ENSG00000137574	trimethylguanosine synthase homolog
MRPL15	ENSG00000137547	mitochondrial ribosomal protein L15
LYPLA1	ENSG00000120992	lysophospholipase I
LYN	ENSG00000147507	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
SEC11B	ENSG00000164738	SEC11 homolog B (S. cerevisiae)
CYP7B1	ENSG00000172817	cytochrome P450, family 7, subfamily B, polypeptide 1
CYP7A1	ENSG00000167910	cytochrome P450, family 7, subfamily A, polypeptide 1
CRH	ENSG00000147571	corticotropin releasing hormone
CHD7	ENSG00000171316	chromodomain helicase DNA binding protein 7
CHCHD7	ENSG00000170791	coiled-coil-helix-coiled-coil-helix domain containing 7
BHLHB5	ENSG00000180828	basic helix-loop-helix domain containing, class B, 5
SULF1	ENSG00000137573	sulfatase 1
SGK3	ENSG00000104205	serum/glucocorticoid regulated kinase family, member 3
RPL7	ENSG00000147604	ribosomal protein L7

Table 6.2 Continued.

Symbol	Ensembl gene	Name/Description
VCPIP1	ENSG00000175073	valosin containing protein (p97)/p47 complex interacting protein 1
UBE2W	ENSG00000104343	ubiquitin-conjugating enzyme E2W (putative)
NCOA2	ENSG00000140396	nuclear receptor coactivator 2
MYBL1	ENSG00000185697	v-myb myeloblastosis viral oncogene homolog (avian)-like 1
MSC	ENSG00000178860	musculin (activated B-cell factor-1)
KCNB2	ENSG00000182674	potassium voltage-gated channel, Shab-related subfamily, member 2
CPA6	ENSG00000165078	carboxypeptidase A6
COPS5	ENSG00000121022	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)
ARFGEF1	ENSG00000066777	ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited)
ADHFE1	ENSG00000147576	alcohol dehydrogenase, iron containing, 1

The following sections contain a discussion of the QTL found in the region of interest, followed by a discussion of the most likely candidate genes.

6.2 Phenotypes in the region

The co-ordinated changes in nutrient partitioning that occur during early lactation in dairy cows were discussed in Chapter 2 and were summarised in a diagram that has been replicated in the current chapter (Figure 6.1). The QTL that are in the region of interest on BTA14 have relevance to particular aspects of control of nutrient partitioning that occur during early lactation (Figure 6.1). Circulating IGF-I concentrations are decreased during early lactation. Changes in circulating IGF-I concentrations play an important role in increasing the availability of nutrients for milk synthesis and secretion. Therefore, the baseline IGF-I QTL may be related to the 60 day milk yield QTL that are also found in the region of interest on BTA 14. The QTL found in this region may be genetically correlated, which would require that there is either pleiotropy or genetic linkage (Chapter 5). However, it is also possible that the QTL of interest are not genetically correlated and occur in the same region of BTA 14 by chance.

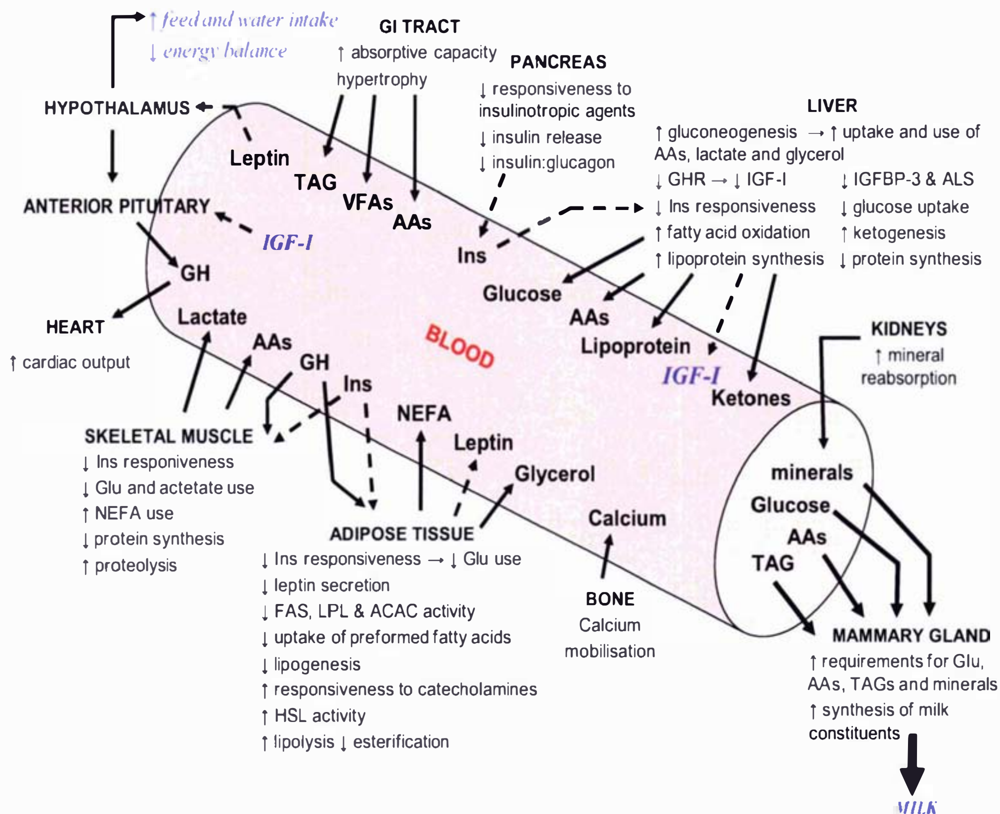


Figure 6.1 Schematic representation of some of the metabolic changes that occur in the periparturient dairy cow. Abbreviations: Ins, insulin; Glu, glucose; FAS, fatty acid synthase; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase; AAs, amino acids; TAG, triacylglycerol; GI tract, gastrointestinal tract; IGF-I, insulin-like growth factor I; GHR, growth hormone receptor; GH, growth hormone; IGFBP-3, Insulin like growth factor binding protein-3; ALS; acid labile subunit; ACAC, acetyl CoA carboxylase; ↑, increase; ↓, decrease; →, which results in. Fluxes: bold line, flux increased; dashed line, flux decreased. The components of the diagram that are in blue relate to the QTL found in the region of interest on BTA14.

With respect to the baseline plasma IGF-I concentration QTL, the most obvious gene to look for would be the IGF-I gene. The IGF-I gene is not present in the list of 105 genes (Appendix G), and it is known that the IGF-I gene is present on BTA5 (De Donato *et al.* 2003). Thus, the next step is to look for indirect indicators or other mechanisms that are known to influence circulating concentrations of IGF-I. IGF-I is produced in most tissues in the body, and exerts biological effects on most cell types and acts by autocrine, paracrine and endocrine mechanisms (Cohick and Clemmons 1993, Thissen *et al.* 1994). Circulating IGF-I is synthesised and secreted primarily by the liver (Cohick and Clemmons 1993, Sjögren *et al.* 1999) IGF-I synthesis is influenced by of a number of factors including GH, nutrition, insulin, T_3 and T_4 and glucocorticoids (McCusker 1998). Low GH concentration, or activity, results in low IGF-I

concentrations, low insulin concentrations are also associated with low IGF-I secretion (McCusker 1998, Thissen *et al.* 1994).

Many studies have demonstrated that energy balance in high producing dairy cows becomes negative at parturition with the onset of milk synthesis and secretion (Bell 1995, Block *et al.* 2001, de Vries and Veerkamp 2000, Van den Top *et al.* 2005). In high producing dairy cows the rate at which consumption of nutrients, required for maintenance and lactation, increases lags behind the rate at which nutrient requirements increase, resulting in reliance on body reserves to meet the deficit in requirements (Lucy *et al.* 2001, Spicer *et al.* 1990). An experiment in dairy cows from weeks 1 to 26 of lactation showed milk yield was positively correlated with feed intake, live weight immediately post-calving, and liveweight loss over the lactation period (Neilson *et al.* 1983). Thus, it would seem the ability of an animal to increase intake as well as mobilise body reserves both contribute to the greater milk yield.

The period of negative energy and protein balance in early lactation is accompanied by a drastic decrease in circulating concentrations of IGF-I and insulin and increased plasma GH and NEFA concentrations (Ronge *et al.* 1988, Vicini *et al.* 1991). There is a gradual increase in IGF-I concentrations as lactation progresses (Abribat *et al.* 1990, Rhoads *et al.* 2004, Vega *et al.* 1991). Post-partum IGF-I concentrations have negative phenotypic correlations with milk production, protein yields (Lacasse *et al.* 1994) and fat-corrected milk yield (Ronge *et al.* 1988). In weeks 2-10 post-partum, IGF-I concentrations were lower in dairy cows that had a high genetic merit for milk fat, protein and yield and higher cumulative milk production over the first 120 days of lactation ($P<0.05$) compared with cows that had an intermediate genetic merit for these milk production traits (Snijders *et al.* 2001). Phenotypic correlations between mean IGF-I concentrations and milk production, calculated for the FJXB animals in the current study, were moderate to low, but more importantly all were negative (Harris *et al.* 2005). Circulating IGF-I concentrations appear to be influenced by nutritional status of the animal; a positive relationship between plasma IGF-I concentration and energy balance has been reported in lactating dairy cows (Cisse *et al.* 1991, Ronge *et al.* 1988, Spicer *et al.* 1990). During early lactation, high

milk yield is associated with low serum IGF-I concentrations and decreased energy balance, therefore greater nutritional stress (Spicer *et al.* 1990). Low insulin and IGF-I concentrations at the onset of lactation may be related to a decrease in the drive to partition nutrients to tissues other than the mammary gland (Spicer *et al.* 1990).

Lactose concentrations in milk are tightly controlled in dairy cows, milk protein shows some slight variation whereas fat concentration is much more variable (Akers 2002, Jenkins and McGuire 2006). The metabolic adaptations that allow this greater variability in fat concentration are unclear but may be related to the ability to mobilise significant amounts of triacylglycerol from body reserves. It is important to note that there were no QTL for milk fat yield in the region of interest on BTA14. This may suggest that because the QTL for 60 day milk, lactose and protein yield are located together in this region that their regulation is coordinated in some way, possibly through protein metabolism. It may be that genes in this region are involved in regulation of protein metabolism and are modified by the action of IGF-I (Liu *et al.* 2006). Or IGF-I may be an indicator of feed intake, energy balance and efficiency of feed utilisation as shown in a number of animal feeding experiments (Breier *et al.* 1986, Elsasser *et al.* 1989, Hegarty *et al.* 2006, Hua *et al.* 1993, Johnston *et al.* 2002, Lapierre *et al.* 1992, León *et al.* 2004, Rausch *et al.* 2002, Ronge and Blum 1989). It is possible that animals with the favourable allele for growth QTL, which are also found in this region (Kneeland *et al.* 2004, Mizoshita *et al.* 2004), have greater protein reserves that can then be mobilised during early lactation and used for milk protein synthesis or gluconeogenesis. These suggested mechanisms could have important roles in determining lactation yield particularly in early lactation.

The objectives of this chapter are to investigate the function of the genes in Table 6.2 and to determine putative candidate genes. Genes that are most likely to be involved in genetic control mechanisms influencing baseline IGF-I, milk, lactose, crude protein, total protein, casein and whey protein yields in the first 60 days of lactation will be selected as putative candidate genes.

6.3 Candidate genes

The function of the genes discussed in this section may be related to the QTL for the mean concentration of plasma IGF-I or the 60 day milk, lactose and protein QTL. It is possible that the IGF-I QTL is an indicator of energy balance or feed intake. Therefore candidate genes that may be involved in control of feed intake or energy balance are discussed. Genes that may be involved in protein or glucose metabolism will also be considered in relation to the aforementioned early lactation milk QTL.

6.4 Protein metabolism candidate genes

During early lactation the demand for nutrients increases rapidly due to the large increase in milk synthesis and secretion (de Vries and Veerkamp 2000, Van den Top *et al.* 2005). Nutrient intake does not keep pace with demand resulting in high-producing dairy cows often being in negative energy balance in early lactation (Spicer *et al.* 1990). Mobilisation of protein reserves is relatively small in comparison to fat mobilisation. Even so, it has been suggested that protein mobilisation is an important process that provides essential amino acids for protein synthesis in the mammary gland or for use as glucose precursors for gluconeogenesis in the liver (Bell *et al.* 2000, Bequette *et al.* 1998, Chilliard 1999, Schei *et al.* 2007). Large differences between cows in the amount of body protein mobilisation that they achieve in early lactation have been reported (Tamminga *et al.* 1997). Another study found that the proportion of carbon in milk casein and lactose derived from body protein was greater in high genetic merit than low genetic merit cows (Wilson *et al.* 1988). Thus, genes that may be involved in the mobilisation of body protein reserves were considered as possible candidate genes for the QTL in the region of interest. Nevertheless, the QTL in the region of interest may not be related to supply of precursors to the mammary gland but instead may be related to the ability of the animal to synthesise proteins. Therefore genes that may be involved in the synthesis of proteins were also considered.

The genes that will be discussed in this section are:

- Ribosome biogenesis regulator homolog (S. cerevisiae) (RRS1),
- Ribosomal protein L7 (RPL7),
- Ribosomal protein S20 (RPS20),
- Mitochondrial ribosomal protein L15 (MRPL15),
- Ubiquitin-conjugating enzyme E2W (putative) (UBE2W) and
- COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis) (COPS5).
- Tripartite motif-containing 55 (TRIM55)
- Musculin (activated B-cell factor-1) (MSC)

They have been selected because they appear to be related to some aspect of protein metabolism. The currently known function of each of the gene products and the relevance they have to the QTL will be discussed in the following sections.

Ribosomal protein L7 (RPL7) and ribosomal protein S20 (RPS20)

Ribosomes are complex macromolecules and are the protein-synthesising organelles responsible for translation and therefore construction of every protein in all living cells. In the case of eukaryotic cells, ribosomes consist of a small (40S) and a large (60S) ribosomal subunit (Liljas 2004). These two subunits are built from about 80 ribosomal proteins and 4 ribosomal RNAs (rRNA) (Brodersen and Nissen 2005, Zemp and Kutay 2007). The rRNAs are not translated but are large molecules that form the core of the ribosomal subunits (Liljas 2004). The two subunits exist separately in the cytosol and when a strand of RNA binds to the small subunit this enables the small and large subunit to bind and form the ribosome. Ribosome assembly in eukaryotic cells is another level of control of gene expression (Ko *et al.* 2006). More than 150 non-ribosomal proteins are involved in the complex process of ribosome biogenesis, which includes

the formation of the constituents of the ribosome subunits, their assembly, and their transport to the sites of protein synthesis. (Zemp and Kutay 2007).

There is a large rRNA molecule contained in each of the ribosomal subunits, which provides the binding sites for ribosomal proteins. The ribosomal proteins stabilise the rRNA and confer its functional three-dimensional structure (Liljas 2004). Crystal structure analyses of ribosomes have demonstrated that a number of ribosomal proteins were located in positions of functional importance, for example, L11 is involved in translation factor binding (Wilson and Nierhaus 2005). Difficulty in attributing a specific function, in terms of ribosomal translational activity, to individual ribosomal proteins is primarily due to the highly cooperative nature of the interactions between rRNA and ribosomal proteins and between ribosomal proteins (Wilson and Nierhaus 2005). The two genes encoding ribosomal proteins found in the region of interest in this investigation are ribosomal protein L7 (RPL7) and ribosomal protein S20 (RPS20)

The RPL7 gene encodes a ribosomal protein that is a structural constituent of the 60S subunit. Thus, it is involved in RNA binding, protein binding and also has transcription regulation activity. Current evidence suggests that the RPL7 protein is essential for making a functional ribosome (Ko *et al.* 2006). The protein can inhibit cell-free translation of mRNAs, suggesting that it plays a regulatory role in the translation apparatus. These authors also suggested that RPL7 may be involved in ribosome assembly and that maturation of the large ribosomal subunit might involve the membrane binding property of RPL7 (Ko *et al.* 2006).

RPS20 is a constituent of the 40S subunit and as a result is involved in translation regulation, RNA binding and protein biosynthesis. An experiment in which individual small subunit ribosomal proteins were deleted from the bacterial *Escherichia coli* (*E. coli*) chromosome showed that RPS20 was one of 6 ribosomal small subunit proteins that were not essential for cell growth (Bubunenko *et al.* 2007). Conversely, in a study of medulloblastomas (a common malignant brain tumour of childhood) in 64 humans, expression levels of RPS20 were found to be negatively associated with survival of patients (De Bortoli *et al.* 2006).

There is a dramatic increase in anabolic processes in the mammary gland that occur around the time of onset of copious milk synthesis and secretion. Because the majority of bovine mammary gland secretory tissue proliferates during gestation when mammary growth is exponential (Capuco and Ellis 2005, Knight *et al.* 1994), to achieve increased milk production after parturition milk secretory activity per mammary epithelial cell must increase (Capuco and Ellis 2005).

The majority of milk proteins are specific proteins unique to lactation (Larson 1985). mRNA for milk-specific proteins are transcribed from chromosomal DNA and are then translated on ribosomes that are attached to the rough endoplasmic reticulum in mammary secretory cells (Holmes *et al.* 2002, Mercier and Gaye 1982). There was a four-fold increase in the fractional rate of mammary protein synthesis in lactating compared with non-lactating dairy goats and it has been suggested that this is indicative of changes in transcriptional regulation (Baracos *et al.* 1991, Toerien and Cant 2007). Parenchymal RNA content and mass were greater in late lactation compared with non-lactating dairy cows representing a greater quantity of translational machinery. In addition the mRNA translation rate was estimated to be 4-fold higher in the lactating mammary tissue (Toerien and Cant 2007). It has been suggested that post-transcriptional mechanisms play a key role in governing milk protein expression. This is supported by findings that transgenic dairy heifers, with additional copies of the genes encoding bovine β- and κ-casein, only exhibited an 8-20% increase in β-casein, however, there was a twofold increase in κ-casein concentration (Brophy *et al.* 2003, Moshel *et al.* 2006).

In a study investigating hepatic gene expression during the dry and early lactation periods of high producing dairy cows, 62 genes were identified as being differentially expressed in relation to physiological state, none of these were ribosomal proteins (Loor *et al.* 2005). However, they found that the expression pattern of the gene that encodes eukaryotic translation initiation factor 4B (eIF4B) was up-regulated from day 1 until day 28 of lactation (Loor *et al.* 2005). eIF4B regulates the activity of eIF2 which mediates the first rate-limiting step of mRNA translation initiation. This step involves binding of initiator methinyl-tRNA to the small ribosomal subunit

(Vary and Lynch 2007). eIF4B stimulates the activity of eIF4A, a subunit of the eIF4F complex that directs the translation machinery to the 5'-end of the mRNA (Raught *et al.* 2001, Shahbazian *et al.* 2006). These are steps that lead to recruitment of the large ribosomal subunit to form a functional ribosome (Vary and Lynch 2007). Another experiment compared gene expression profiles of lactating bovine mammary tissue with non-lactating tissue on a bovine mammary gland microarray. There were no genes encoding ribosomal proteins that were identified as being up-regulated in the lactating mammary gland (Suchyta *et al.* 2003). However, eIF4G (a subunit of the eIF4F complex) expression was found to be decreased compared with non-lactating mammary tissue (Suchyta *et al.* 2003). This seems unusual considering the higher rate of mRNA translation that has been observed even in late-lactating compared with non-lactating mammary glands (Toerien and Cant 2007). In a more recent experiment, expression of the eIF4E gene (the third subunit of the eIF4F complex) was found to increase dramatically by day 15 of lactation and remained elevated until day 240 of lactation (Bionaz and Loor 2007b). Thus, it would seem that increased expression of the eukaryotic translation initiation factors plays a key role in increasing the activity of ribosome in response to lactation. Further evidence supporting the hypothesis that the expression of genes encoding ribosomal proteins is not an important limiting factor in lactation is provided by an experiment that investigated nine genes for use as internal control genes. Ideal internal control genes have a relatively constant expression in the mammary gland throughout the lactation cycle. After correction for the dilution effect caused by increased RNA concentration in the mammary gland, RPS9 and RPS15 were found to have stable gene expression across the lactation cycle (Bionaz and Loor 2007a). It is possible that if the expression of these two genes encoding ribosomal proteins is not increased, genes encoding other ribosomal proteins, such as RPS20 and RPL7, would not be increased either.

It seems likely that protein synthesis in the lactating mammary gland is increased by acceleration of mRNA translation initiation rather than transcription of the individual ribosomal

protein genes. Thus, the genes that encode RPS20 and RPL7 are unlikely to be the genes responsible for the QTLs present in the region of interest on BTA14.

Ribosome biogenesis regulator homolog (S. cerevisiae) (RRS1)

Ribosome biogenesis regulator homolog (*S. cerevisiae*), as the name suggests, is a gene that is homologous to the yeast (*Saccharomyces cerevisiae*) RRS1 regulator of ribosome synthesis 1 (Rebhan *et al.* 1997). A study in yeast showed that the protein product of RRS1 is localised in the nucleoplasm and the nucleolus (Tsuno *et al.* 2000). In cells that the RRS1 protein was depleted, precursor rRNA processing was reduced and the appearance of the 60S ribosomal subunit was reduced compared with the 40S subunit, suggesting this protein has a role in maturation of certain precursor rRNAs and 60S subunit assembly (Tsuno *et al.* 2000). The RRS1 protein also recruits ribosomal protein L11 and a molecule that is a complex of the ribosomal protein L5 and a precursor rRNA to the 60S precursor subunits, which is a necessary step in the processing of the large subunit precursor (Nariai *et al.* 2005). Another experiment demonstrated that the RRS1 protein stays on the precursor 60S particle from early to very late stage maturation and is required for export of the 60S ribosomal subunit from the nucleus to the cytoplasm (Miyoshi *et al.* 2004).

The functional protein derived from the human RRS1 homologue has been purified from the nucleoli and metaphase chromosomes from human cell lines (Scherl *et al.* 2002, Uchiyama *et al.* 2005). It has been suggested from results of RNA interference studies that RRS1 may have a functional role in chromatid cohesion. Chromatid cohesion is a chromosomal protein complex that uses a large proteinaceous ring to tie sister chromatids together to allow pairs to be recognised and aligned during mitosis and distributed correctly during anaphase (Fukui *et al.* 2006, Uhlmann 2004). RRS1 has also been implicated in a number of disease states including Huntington's disease and is induced by influenza virus replication (Nariai *et al.* 2005).

The functional protein of the RRS1 gene has been implicated in mitosis and viral replication (Fukui *et al.* 2006, Nariai *et al.* 2005, Uhlmann 2004), therefore, it is possible that RRS1 may be

up-regulated during cell proliferation in the mammary gland. The majority of bovine mammary gland secretory tissue proliferates during gestation when mammary growth is exponential (Capuco and Ellis 2005, Knight *et al.* 1994). Processes of proliferation and cell turnover have been shown to accelerate from approximately 77 days pre-partum and are highest around the time of parturition (Capuco *et al.* 1997, Sorensen *et al.* 2006). Milk yield is a function of the number and secretory activity of mammary epithelial cells (Capuco and Ellis 2005). It has been suggested that selection for increased milk production promotes both increased cellular differentiation and increased cell proliferation (Akers *et al.* 2006). Thus it is possible that RRS1 may be more strongly upregulated resulting in greater cell proliferation in cows with higher milk production.

Alternatively, the RRS1 functional protein is important in the processing of the 60S ribosomal subunit in yeast (Tsuno *et al.* 2000). Comparison of the protein product of RRS1 sequence with database sequences showed that the protein is conserved throughout eukaryotes (Tsuno *et al.* 2000). However, sequence similarity does not necessarily mean that there will be functional similarity (Gerlt and Babbitt 2000, Wilson *et al.* 2000). To confirm the function of a gene precisely, requires investigation of both its sequence and expression characteristics (Zhou and Gibson 2004). By way of illustration, experiments have been conducted that identified a set of co-expressed genes, known to be associated with a particular function, in one organism. A set of sequence homologs were then identified in another organism and their expression measured, only a subset of the genes were co-expressed in the second organism. The genes that were not co-expressed were not considered to be functionally conserved homologs (Bergmann *et al.* 2004, Zhou and Gibson 2004). Thus, functional assignment based on homology alone should be considered with caution (Gerlt and Babbitt 2000). Nevertheless, it is possible that the RRS1 gene may be important for processing of the 60S ribosomal in bovine cells. After parturition, the initial rise to peak milk production is primarily due to increased milk secretory activity per mammary epithelial cell (Capuco and Ellis 2005). There is a dramatic increase in anabolic processes in the mammary gland that occur around the time of onset of copious milk synthesis

and secretion. For example, *in vitro* studies of mammary tissue showed a 280% increase in α-lactalbumin release (103 ng/mg to 289 ng/mg) from day 260 of gestation to day 49 of lactation (Keys *et al.* 1989). Up-regulation of RRS1 may result in a greater maturation rate of the 60S ribosomal subunit. Thus, more 60S ribosomal subunits would be available to bind to the 40S ribosomal subunit to form functioning ribosomes and subsequently translate and synthesise the proteins necessary for milk production. Perhaps cows with a greater propensity for transcription and translation of the RRS1 gene in early lactation have a greater proportion of 60S subunits in a mature state, which may result in more matures ribosomes and production of greater quantity of proteins required for milk production.

Mitochondrial ribosomal protein L15 (MRPL15)

Mammalian mitochondrial ribosomal proteins are encoded by nuclear genes and help in protein synthesis within the mitochondria. Mammalian mitochondria synthesise 13 proteins (in humans) from mitochondrial DNA within the inner mitochondrial membrane (Smits *et al.* 2007). These proteins are components of complexes that are essential for oxidative phosphorylation and synthesise approximately 90% of the ATP in eukaryotic organisms. Mitochondrial ribosomes (mitoribosomes) are made up of ribosomal proteins and ribosomal RNA (rRNA) that contribute to the structure and function of the two ribosomal subunits (Wilson and Nierhaus 2005), a small 28S subunit and a large 39S subunit (Koc *et al.* 2001).

MRPL15 encodes a 39S subunit protein and is likely to be involved in protein synthesis and translational regulation. MRPL15 may be a methyltransferase and may bind S-adenosyl-L-methionine and RNA (Wilson and Nierhaus 2005). Among different species, the proteins comprising the mitoribosome differ greatly in sequence, and sometimes in biochemical properties, which prevents easy recognition by sequence homology.

The number of mitochondria increase in mammary epithelial cells with the onset of lactation. They are the site of production of ATP by the process of oxidative phosphorylation, and generation of precursors for synthesis of non-essential amino acids and fatty acids (Akers 2002).

Currently there are no reports in the literature about the function of the protein encoded by MRPL15 or changes in its expression in relation to different physiological states. In an experiment that corrected for the dilution effect caused by increased RNA concentration due to lactation, there was a significant effect of time on MRPL39 expression. Indicating that the MRPL39 gene had differential transcriptional regulation during the lactation cycle (Bionaz and Loor 2007a). If there is co-ordinated control of transcription of mitochondrial ribosomal proteins, it is possible that a similar pattern of differential transcriptional regulation may occur for the MRPL15 gene during lactation. The crucial role that mitochondria play in energy metabolism within the cell means that they are potential candidates for influencing milk production. However, if the MRPL15 gene was affecting the energy generated by mitochondria and therefore milk production, it would likely affect milk fat as well as milk protein and lactose. Since there are no milk fat QTL currently known in the region of interest in this investigation an effect of this gene on milk, lactose and protein yields does not seem likely. However, as new information becomes available about the function and expression of MRPL15, it may become clearer if further research on this gene in relation to milk production is required.

Ubiquitin-conjugating enzyme E2W (putative) (UBE2W)

The UBE2W gene product is an ubiquitin-conjugating enzyme or ubiquitin-carrier protein (E2) that is involved in the second step of ubiquitin conjugation in the ubiquitin-proteasome pathway (UPP)(Attaix *et al.* 1998). The UPP is the major system responsible for proteolysis in cells and is required for targeted degradation of most short-lived proteins in the eukaryotic cell (Genini and Catapano 2006).

The sole ubiquitin-activating enzyme (E1) hydrolyses ATP and activates ubiquitin, an abundant and highly conserved 76 amino acid polypeptide (Attaix *et al.* 1998, Ciechanover *et al.* 2000). Ubiquitin-conjugating enzymes (E2s) transfer the activated ubiquitin from E1 to a member of the ubiquitin-protein ligase family (E3). E2s function with E3s to mediate covalent binding of ubiquitin to target proteins (Attaix *et al.* 1998, Back 2003, Genini and Catapano 2006, Wing 2003). E3s play a role in the selection of proteins for conjugation and catalyse the formation of

polyubiquitin chains on target proteins (Attaix *et al.* 1998). Polyubiquitin chains are recognised by 26S proteasomes as a degradation signal and as such result in degradation of the polyubiquitylated proteins by the 26S proteasome (Attaix *et al.* 1998, Ciechanover *et al.* 2000). Ubiquitylated proteins can be rescued prior to proteasome degradation by the rapid removal of ubiquitin chains catalysed by deubiquitinating enzymes (Wing 2003). Thus, these cycles of ubiquitylation-deubiquitination reactions can rapidly modulate protein level and activity (Genini and Catapano 2006).

The E2s are a superfamily of related proteins with a molecular weight range of 14 to 35 kDa (Attaix *et al.* 1998). The UniProtKB/Swiss-Prot entry indicates that the unprocessed precursor of UBE2W is 151 amino acids in length and has a molecular weight of 17 kDa. An assessment of the amino acid sequence shows similarity to the ubiquitin-conjugating enzyme (E2) family (http://au.expasy. org/uniprot/Q96B02#ref, 16 March 2007). To date, various E2 species have been found in muscle, these include the 14-, 17- and 20-kDa species of E2 (Attaix *et al.* 1998).

It has been shown in a number of experiments that mobilisation of endogenous protein occurs during early lactation (Chilliard and Robelin 1983, Kokkonen *et al.* 2005, Overton *et al.* 1998, Phillips *et al.* 2003, Wilson *et al.* 1988). Mobilisation of muscle protein in early lactating ruminants possibly results from a decrease in synthesis, but is mostly a result of increased degradation (Baracos *et al.* 1991, Chilliard *et al.* 1998a, Overton *et al.* 1998, Phillips *et al.* 2003, Smith *et al.* 1981, Wilson *et al.* 1988). During a 6-hour hyperinsuliaemic-euglycaemic clamp, the 40-fold increase in circulating insulin concentrations caused a decrease in ubiquitin mRNA in fast-twitch or mixed (fast- and slow-twitch) skeletal muscle but did not affect the abundance of 14-kDa E2 mRNA in the same muscles compared with saline infused control goats (Larbaud *et al.* 1996). Thus, the low circulating insulin concentrations that occur at the onset of lactation in dairy cows (Accorsi *et al.* 2005, Holtenius *et al.* 2003, Komatsu *et al.* 2005) may influence the UPP. Alterations in the UPP may contribute to mobilisation of body protein from skeletal muscle for the purpose of supporting lactation.

The contribution of body proteins to the energy requirements of lactation is small but quantitatively important in the ruminant. Mobilised proteins supply essential amino acids for protein synthesis in the mammary gland or amino acids for gluconeogenesis in the liver (Bell *et al.* 2000, Bequette *et al.* 1998, Chilliard 1999, Schei *et al.* 2007). It has been suggested that the greater persistency of milk production in high genetic merit dairy cows is related to size of body protein reserves and the ability to mobilise them during early lactation to support milk protein and lactose synthesis (Bequette *et al.* 1998). Conversely, it has been suggested that relocation of body protein reserves as a result of hypertrophy of gut and liver tissues may be more important than the contribution to milk protein (Sutter and Beever 2000). Experiments in dairy cows have shown that an increase in dietary protein supply increased milk, protein and lactose yields from week 1 of lactation onwards and some experiments induced greater mobilisation of fatty acids from adipose tissue (de Boer *et al.* 1991, Kokkonen *et al.* 2002, Moussavi *et al.* 2007, Ørskov *et al.* 1981). These findings indicate the limited potential of cows to compensate for a deficit of feed protein supply by mobilising tissue proteins (Kokkonen *et al.* 2002, Ørskov *et al.* 1981), thus supporting the suggestion that amino acids and glucose precursors are limiting to milk production.

It is not clear how important mobilisation of protein reserves is for providing precursors for milk production, however, it is possible that the functional product of the UBE2W gene is important in break down of protein reserves in early lactation. This idea is supported by the fact that the QTL in the region are all milk traits up to day 60 of lactation, not for periods later in lactation. Experimental work conducted to date, indicates that protein breakdown only occurs very early in lactation (Phillips *et al.* 2003, Tamminga *et al.* 1997), consequently UBE2W may play a role in protein breakdown in early lactation.

Alternatively, there is considerable protein turnover within mammary secretory cells including catabolism of signal proteins as well as a significant degradation of intracellular proteins, such as enzymes and structural proteins, and even milk proteins (Bequette *et al.* 1998, Holmes *et al.* 2002). It is thought that protein turnover in the mammary gland is important for providing a

constant supply and the correct balance of amino acids necessary for both milk protein and lactose synthesis (Bequette *et al.* 1998). Perhaps the action of UBE2W is important in the mammary gland, as well as in skeletal muscle, where it may play a role in the ubiquitylation of proteins to target them for degradation. Differential regulation of this gene may result in different rates of protein degradation or perhaps targeting of different proteins for degradation. Perhaps certain genotypes result in more appropriate amino acid concentrations for synthesis of lactose and milk proteins, which would fit with the milk, lactose and protein QTL found in the region. If this were the case it would seem likely the differences between genotypes would continue throughout lactation and since the QTL in this region are for the first 60 days of lactation only, this hypothesis needs to be taken with caution. However, once food intake is sufficient to supply the nutrient requirements of lactation, which usually occurs a few weeks after peak lactation, then the protein degradation processes described in this section may not be as important as they would be in early lactation. This would explain why the QTL in the region of interest are only for the first 60 days of lactation.

COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis) (COPS5)

The COPS5 gene product was first identified as a transcriptional coactivator of AP1 proteins called Jun activating domain binding protein 1 (Jab1) (Claret *et al.* 1996). It is also termed CSN5, as it is the fifth component of the COP9 signalosome complex (CSN), which is made up of eight subunits (CSN1-8) in mammals. CSN5 is possibly the key subunit that integrates the multiple functions of the CSN complex (Wei and Deng 2003). CSN5 is a cytosolic and nuclear protein and has both CSN-associated and CSN-independent activities in UPP protein turnover and signal transduction, respectively (Lue *et al.* 2006).

The broad functions of CSN have been mainly attributed to its role in the UPP mediated protein degradation (Rosel and Kimmel 2006). Ubiquitin ligases termed SKP1-Cullin/CDC53-F-box protein (SCF) complexes catalyse the polyubiquitinylation of proteins to target them for degradation. Cullin subunits are an integral part of SCF and require addition of the ubiquitin-

like protein Nedd8 for its function (Rosel and Kimmel 2006). The CSN complex is an essential regulator of the ubiquitin conjugation pathway by mediating the removal of Nedd8 from the cullin subunits of the SCF-type E3 ligase complexes. CSN5 probably acts as the catalytic centre that mediates the cleavage of Nedd8 from cullins, resulting in cessation of the polyubiquitinylation process. This allows the subsequent degradation of the polyubiquitinylated substrates by proteasomes (Calligé *et al.* 2005, Rosel and Kimmel 2006).

Knockout or mutational studies in a variety of organisms indicate that CSN5 is involved in cell-cycle progression, checkpoint activation, genome stability, and cell survival. CSN5, either as a monomer or part of the CSN complex, plays a critical role in nuclear export and directly interacts with and regulates the activity, stability and degradation of multiple intracellular regulatory molecules, such as c-jun (major component of the AP-1 transcription factor, AP-1 regulates various aspects of cell proliferation and differentiation), p27 (involved in inducing G_1 cell cycle arrest), p53 (transcription factor involved in regulating the cell cycle), Cullin, Smad4 (transcription factor that mediates TGF-β signalling to regulate cell growth and differentiation), MIF (involved in signal transduction that regulates cell functions such as gene expression, proliferation and apoptosis), and HIF1 α (Lue *et al.* 2006, Oh *et al.* 2006, Wang *et al.* 2006). Therefore, CSN5 and the CSN complex are important in the regulation of molecules involved in multiple signalling pathways in various cellular and developmental processes, such as early embryonic development and cell proliferation (Tomoda *et al.* 2004).

From this information it does not seem that the COPS5 gene is a likely candidate for the milk QTL in the region of interest unless it is involved in the cell proliferation process that the mammary gland undergoes during pregnancy and the epithelial cell turnover that continues into lactation (Capuco *et al.* 2001, Sorensen *et al.* 2006). CSN5 interacts with diverse proteins that play roles in many cell processes (Chamovitz and Segal 2001). There is still much to be learnt about the roles of the COPS5 gene product, CSN5, in the cell. It is possible that an as yet unknown function may be important in controlling early lactation milk, lactose and milk protein yields. New functions are still being discovered, for example, two recently reported

experiments have indicated that CSN5 interacts with the α_{1C} subunit of the L-type Ca^{2+} channels and inhibits channel activities in the rat heart (Kameda *et al.* 2006), and CSN is likely to play a role in a phosphorylation event that is necessary for oestrogen receptor α that is complexed with oestradiol and/or a coactivator to bind to target DNA and recruit a preinitiation complex enabling translation of target genes (Calligé *et al.* 2005). Oestrogen is one of the major stimuli for mammary gland growth during pregnancy so it is possible that this recently discovered role of CSN may be important in mammogenesis (Akers 2002, Holmes *et al.* 2002). Further study is required to investigate a role, if any, for this gene in influencing the QTL in the region of interest.

Tripartite motif-containing 55 (TRIM55)

Tripartite motif-containing 55 (TRIM55) is also known as Muscle-specific RING finger protein 2 (MURF-2) or RING finger protein 29 (RNF29) (Zhang *et al.* 2006). Four alternatively spliced transcript variants encoding distinct isoforms have been described for the TRIM55 gene (Zhang *et al.* 2006). TRIM55 may regulate gene expression and protein turnover in muscle cells. *In vitro* studies have shown that the highest levels of TRIM55 expression in skeletal muscle cells occurred early after the onset of differentiation and the TRIM55 protein associates transiently with microtubules, myosin, and titin during muscle sarcomere assembly (Pizon *et al.* 2002). TRIM55 is involved in the establishment and/or rearrangement of cytoskeletal arrays formed during muscle development (McElhinny *et al.* 2004). It has been suggested that TRIM55 may have a signalling role in differentiation prior to cytoskeletal formation and may be involved in transcription during differentiation of myocytes, thus influencing expression or activity of other molecules involved in formation and function of cytoskeletal arrays (McElhinny *et al.* 2004, Pizon *et al.* 2002).

Experiments conducted using skeletal muscle samples collected from pigs at various stages of development showed that TRIM55 expression levels were up-regulated during embryo stages and down-regulated during post-natal stages of development (Zhang *et al.* 2006). Studies using rat tissue demonstrated down regulation of TRIM55 in mature cardiac and skeletal tissues

(Centner *et al.* 2001, Pizon *et al.* 2002). A study in humans, who had developed skeletal muscle wasting as a result of chronic heart failure, were found to have increased TRIM55 and decreased IGF-I and myostatin gene expression compared with healthy controls (Le Jemtel *et al.* 2005). TRIM55 has been mapped to porcine chromosome 4 and BTA 14 and in both species significant QTL affecting growth, muscle and fat deposition and carcass weight have been identified in those regions (Mizoshita *et al.* 2004, Varona *et al.* 2002). Current data on the role of TRIM55 suggests that this gene plays an important role in normal muscle development and is a candidate gene for traits related to skeletal muscle development and further investigation is required (Zhang *et al.* 2006).

A possible role for this gene in lactation may be through influencing mobilisation of muscle protein in early lactation. Dairy cows mobilise body protein reserves in early lactation (section 2.8 and page 143). The mobilisation of these reserves assists in meeting the energy and protein deficits in early lactation by providing amino acids for milk protein synthesis and glucogenic amino acids for gluconeogenesis liver (Bell *et al.* 2000, Schei *et al.* 2007). TRIM55 appears to have a specific role in muscle tissues, therefore, may enable mobilisation of labile protein reserves.

During the peri-partum period and particularly after parturition when the onset of copious milk synthesis and secretion occurs the circulating concentrations of IGF-I are dramatically reduced (Rhoads *et al.* 2004, Ronge *et al.* 1988, Vicini *et al.* 1991). There are some similarities between the mechanisms operating in humans with skeletal muscle wasting and mobilisation of protein reserves in early lactation dairy cows, in both situations IGF-I was decreased either in the circulation or decreased gene expression (Le Jemtel *et al.* 2005, Rhoads *et al.* 2004). Perhaps as is the case in human skeletal muscle wasting, the expression of the TRIM55 gene is increased in the muscle of dairy cows enabling protein mobilisation during early lactation. Interestingly, in an experiment investigating the effect of pre-partum nutrition on hepatic gene expression of Holstein cows during the dry period and in early lactation, TRIM55 was found to be differentially expressed due to pre-partum nutritional treatments (Loor *et al.* 2006).

In a study that exploited the natural differences in the ^{13}C concentration in C_3 and C_4 plants, the proportion of carbon in casein (0.34 vs 0.25) and lactose (0.24 vs 0.11) derived from body reserves was greater in HGM compared with LGM dairy cows in early lactation (Wilson *et al.* 1988). The estimated plasma amino acid pool size was consistently higher in HGM than LGM cows and the authors speculated that plasma amino acid pool size may be a factor that influences milk production (Wilson *et al.* 1988). TRIM55 is likely to play an important role in normal muscle development (Zhang *et al.* 2006), and is a candidate gene for growth QTL in both pigs and cattle (Mizoshita *et al.* 2004, Varona *et al.* 2002). The size of the body protein reserves and the ability to mobilise the reserves may be influenced by the level of expression of the TRIM55 gene and may be important factors influencing 60 day milk, lactose and protein yield QTL.

Musculin (activated B-cell factor-1) (MSC)

Musculin (MSC, also called repressor of myogenesis (MyoR) or activated B-cell factor-1 (ABF-1)) appears to be a transcription repressor (Zhao and Hoffman 2006). MSC is expressed and may play a role in embryogenesis and foetal development (Yu *et al.* 2003). MyoR is expressed as early as the blastocyst stage, 3-3.5 days post coitum, in mouse embryos. Over expression of MyoR inhibits retinoic acid induced differentiation in an embryonal carcinoma cell line and is lethal to early mouse embryos leading the authors to suggest that MyoR has a general role in the repression and/or determination of embryonic cell differentiation (Yu *et al.* 2003). Low level expression of MyoR has been detected in some adult tissues from mice, including the brain, spleen and muscle but not in liver, lung, thymus or kidney (Robb *et al.* 1998). In another experiment MyoR expression was detected in brain, lung, heart, small intestine, spleen and thymus but not in kidney, skeletal muscle, skin, stomach and testis (Yu *et al.* 2003).

MyoD is one of four known myogenic regulators that activate muscle gene expression during both myogenesis (formation of muscular tissue, in particular during embryonic development) and muscle regeneration (Zhao and Hoffman 2006). Adult mice were injected with cardiotoxin into the gastrocnemius muscle on both sides to induce muscle degeneration/regeneration.

MyoD peaked at 12h and then again at 3 days after injection, followed by down-regulation at day 4-5 of muscle regeneration (Zhao *et al.* 2003). Up-regulation of MSC was found to occur at the time of transcriptional down-regulation of MyoD. It has been suggested that MSC is a likely key negative transcriptional regulator of MyoD during muscle regeneration *in vivo* and may bind the MyoD promoter to directly down-regulate MyoD transcription (Zhao and Hoffman 2006). In addition, MSC is down-regulated during regenerative processes in adult mice kidneys (Hishikawa *et al.* 2005) and has high expression levels in activated human B cells (Massari *et al.* 1998).

A possible mechanism by which the MSC gene may be involved in lactation is through its role in down-regulation of muscle regeneration (Hishikawa *et al.* 2005, Zhao and Hoffman 2006). It may be that MSC is up-regulated in muscle tissue during early lactation to prevent amino acids being utilised for muscle protein synthesis. During early lactation in ruminants mobilisation of protein reserves is a result of decreased protein synthesis and increased protein degradation (Baracos *et al.* 1991, Wilson *et al.* 1988). Thus, it is possible that the MSC gene is involved in lactose and protein yields in the first 60 days of lactation, however more research is required to test this hypothesis.

Summary

Eight genes were discussed in this section, which focussed on genes likely to be involved in some aspect of protein metabolism. Following assessment of the currently known functions of each gene, Tripartite motif-containing 55 and Ubiquitin-conjugating enzyme E2W (putative) were considered to be the most suitable candidates because of relevance of their functions to milk production in the first 60 days of lactation. The size of the body protein reserves and the ability to mobilise these reserves may be influenced by the level of expression of the TRIM55 gene. These may be important factors in supply of amino acids for milk protein synthesis and glucogenic amino acids for glucose synthesis in the liver and therefore influence 60 day milk, lactose and protein QTL. Mobilisation of protein reserves may be of particular importance in cows selected for New Zealand farming systems because spring pasture, the vast majority of the

diet in early lactation, does not supply adequate quantities of dry matter or readily fermentable carbohydrate which are associated with glucogenic volatile fatty acids (Waghorn 2002). In addition, it has been suggested that there is an association between TRIM55 and IGF-I concentrations during muscle wasting, which fits well with the IGF-I QTL present in the region of interest in this study. The protein product of UBE2W may also contribute to supply of amino acids for milk protein synthesis and liver gluconeogenesis through mobilisation of labile protein reserves and protein turnover in mammary secretory cells. Perhaps up-regulation of the UBE2W gene occurs in both skeletal muscle and the mammary gland increasing proteolysis in both tissues thus contributing to milk, lactose, crude protein, total protein, casein, and whey protein yields in the first 60 days of lactation.

6.5 Control points in metabolic pathways

The genes in this section may play a role in some aspect of a metabolic pathway important in lactation. Many changes occur in metabolic pathways in the peri-parturient period to enable the dairy cow to synthesise and secrete milk (Chapter 2). For example, in adipose tissues there are changes in a number of enzymes. Activities of fatty acid synthetase, lipoprotein lipase, glucose-6-phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase were decreased in early lactation (Chilliard *et al.* 1991b, Shirley *et al.* 1973). Meanwhile an increase in the number of β-adrenergic receptors per cell (Jaster and Wegner 1981) contributes to an increase in lipolysis and a decrease in lipogenesis. There are also changes in circulating hormone concentrations ((Abribat *et al.* 1990, Rhoads *et al.* 2004, Ronge *et al.* 1988, Vicini *et al.* 1991) and increased synthesis and secretion of the constituents of milk within the mammary gland (Keys *et al.* 1989, Neville *et al.* 2002). These processes are likely to have control points at a cellular level, therefore, genes that may be involved in any of the vast number of changes in metabolic and physiological pathways that occur to support lactation were investigated.

The genes that will be discussed are:

- Nuclear receptor coactivator 2 (NCOA2),

- Serum/glucocorticoid regulated kinase family, member 3 (SGK3),
- Regulator of G-protein signalling 20 (RGS20/RGSZ1),
- RAB2, member RAS oncogene family (RAB2),
- ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited) (ARFGEF1) and
- ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H (ATP6V1H).

The currently known functions and how these functions may be important in the production of milk, lactose and milk proteins will be discussed for each of the genes in this section.

Nuclear receptor coactivator 2 (NCOA2)

Nuclear receptor coactivator 2 (NcoA2) is also known as transcriptional intermediary factor-2 (TIF-2), glucocorticoid receptor interacting protein-1(GRIP-1), and steroid receptor coactivator-2 (SCR-2) (Picard *et al.* 2002, Xu and Li 2003). NcoA2 is a member of the p160 family of transcriptional coactivators (SRCs) for nuclear receptors (NRs) (Jeong *et al.* 2006). These coactivators interact with histone acetyltransferases and methyltransferases to recruit them to specific promoter regions in a ligand-dependent manner. NcoA2 is able to interact with multiple NRs in a ligand-dependent manner and significantly enhance NR-dependent transcription. NcoA2 can also interact with and coactivate other transcription factors. On its own and as part of transcription factor complexes, NcoA2 stimulates chromatin remodelling, recruits general transcription factors, and ultimately enhances gene expression (Xu and Li 2003). NcoA2 and the other SRCs are involved in an extremely complex system, integrating multiple incoming stimuli into an appropriate cellular response by controlling up and down regulation of transcription activation (McKenna and O'Malley 2002, Rogatsky *et al.* 2001). Coactivators may also be the factors that mediate many downstream NR-related events (McKenna and O'Malley 2002).

The cellular balance of coactivators and corepressors affords a smooth and tightly controlled induction curve for NR-mediated gene expression. Increased coactivator would likely provide a more rapid transcription response. NcoA2 has also recently been shown to change EC_{50} (half maximal effective concentration), which may be of considerable importance for responding to changes in physiological state. Coactivator/corepressor levels in different individuals might explain, at least in part, some of the variability in individual phenotypes observed throughout species populations (McKenna and O'Malley 2002). Given that coactivators act at the amplification step in gene expression, a few percent difference in the inherited coactivator levels in the population could underlie major alterations in hormone-mediated development of, for example, the musculo-skeletal and organ systems (McKenna and O'Malley 2002).

NcoA2 is widely expressed, its mRNA has been detected in many tissues including the placenta, testis, brain, heart, liver, pancreas and uterus, expression levels are tissue and cell-type dependent (Xu and Li 2003). Studies conducted with NcoA2 null mice have demonstrated that both male and female adult NcoA2 null mice had low fertility compared to wild type mice (Géhin *et al.* 2002, Mukherjee *et al.* 2006). NcoA2 also had an essential role in progesterone-induced mammary ductal side-branching and alveologenesis, cellular processes that normally occur with pregnancy onset (Mukherjee *et al.* 2006).

NcoA2 null mice were resistant to obesity, despite them eating more than the wild-type mice (Picard *et al.* 2002). Whole body insulin sensitivity appeared to be enhanced in the absence of NcoA2. Thus, NcoA2 null mice had lower fasting glycemia, lower one hour postprandial insulin concentration despite similar glucose, and higher insulin stimulated glucose clearance. They also had lower fasting triglyceride concentrations. Lipolysis was higher in NcoA2 null mice cells under both basal conditions and after exposure to adrenaline. They had lower levels of antilipolytic proteins, lower expression of genes involved in fatty acid uptake, lower leptin mRNA levels in adipose tissue and lower plasma leptin concentrations. Altered gene expression due to absence of NcoA2 enhanced lipolysis and impaired fat uptake and storage (Picard *et al.* 2002). In the livers of NcoA2 null mice, glucose transporter 2 (GLUT2) and

glucokinase (Gck) gene expression were decreased in the NcoA2 null mice. These two enzymes catalyse the initial step in the use of glucose by β-cells and liver at physiological glucose concentrations (Jeong *et al.* 2006).

Thus, NcoA2 is involved in the control of uptake and storage of glucose. Fatty acid degradation and glycolysis pathways are up-regulated and fatty acid, cholesterol, and steroid biosynthesis pathways are down-regulated in the liver of NcoA2 null mice (Jeong *et al.* 2006). These results are consistent with the phenotype observed in NcoA2 null mice, having increased energy expenditure (Jeong *et al.* 2006, Picard *et al.* 2002). NcoA2 null mice had an overall down-regulation of target genes, indicating a largely promotional activity on target gene expression (Jeong *et al.* 2006).

There are two possible ways the NcoA2 gene may influence the QTL in the region of interest in this study. Firstly, by affecting mammogenesis during pregnancy or secondly, by influencing changes in the metabolism of tissues that are important in nutrient partitioning in early lactation. NcoA2 has an essential role in progesterone-induced mammary ductal side-branching, alveologenesis and epithelium proliferation in the mouse during pregnancy (Mukherjee *et al.* 2006). The amount of tissue in the bovine mammary gland is subject to exponential growth during pregnancy. The increase in the first half of pregnancy is mainly an extension of the duct system and proliferation of the supporting tissues, while in the 5th to 6th month of pregnancy alveoli appear and increase in number throughout the remainder of pregnancy (Holmes *et al.* 2002). The concentrations of oestrogen and progesterone are simultaneously elevated during much of gestation in cows, particularly during the later portion of gestation and are essential for much of the mammary growth during gestation (Holmes *et al.* 2002, Schams *et al.* 2003). It is possible that the NcoA2 gene product plays a similar role in mammogenesis during pregnancy in the bovine mammary gland as it does in the mouse. Differential expression of the gene may influence the extent of mammary gland tissue growth, which in turn may affect milk production, a relationship that has been suggested in previous studies (Akers *et al.* 2006, Capuco and Ellis 2005).

Fatty acid mobilisation from adipose tissue has a pivotal role in supplying energy in the lactating dairy cow (McNamara 1995). It is apparent from the experimental work conducted with NcoA2 null mice that this gene has an important role in energy homeostasis (Picard *et al.* 2002). Since adaptations that occur in energy metabolism, around the time of onset of copious milk synthesis and secretion, play a key role in early lactation in the high producing dairy cow, it seems possible that the NcoA2 gene has a role in these adaptations. NcoA2 may be down-regulated in the liver and adipose tissue of dairy cows resulting in decreased glucose uptake and increased energy generation by the liver, and increased lipolysis in adipose tissue as seen in NcoA2 null mice (Picard *et al.* 2002). During the period of negative energy balance that usually occurs in early lactation, NEFAs are released from adipose tissue and contribute substantially to oxidative energy metabolism in non-mammary tissues, which spares glucose and amino acids for use in the mammary gland (Chilliard *et al.* 2000, Vernon 1998). Animals of higher genetic merit for milk production have higher rates of lipolysis compared with low genetic merit cows (McNamara and Hillers 1986b) and milk yield was positively correlated with live weight loss during lactation (Neilson *et al.* 1983). Further evidence is provided by an experiment in which a hyperinsulinaemic-euglyceamic clamp was administered to dairy cows during week two of lactation. The clamp caused a 68% reduction in plasma NEFA, a 27% reduction in milk fat yield and the milk fat was composed of a lower proportion of long chain fatty acids (Corl *et al.* 2006). This demonstrated the important contribution that mobilisation of fatty acids from body fat reserves makes to milk fat yield and concentration in early lactation (Corl *et al.* 2006).

If the NcoA2 gene was differentially regulated during the onset of lactation then it would be reasonable to expect an affect on milk fat yield as well as the other milk components. However, there were no milk fat yield QTL identified in the region of interest in this study. On the other hand, NcoA2 was involved in several aspects of energy homeostasis in the mouse and it is possible that the various functions of this gene combine to improve the availability of precursors for milk proteins and lactose. Alternatively, the functional protein encoded by this gene is a member of a family of coactivators/corepressor, and may play a role in lactation in this capacity.

For example, NcoA2 may increase the expression of a gene or genes in response to a particular stimulus during parturition or the onset of lactation, which in turn could impact on as yet unidentified pathways important to milk production. NcoA2 may act at the amplification step in gene expression and a few percent difference in the inherited coactivator levels in the population could underlie major alterations in hormone-mediated changes in physiology (McKenna and O'Malley 2002). The gene(s) that NcoA2 acts upon could either be in close proximity on the chromosome but it does not have to be (Chapter 5). If the genes were not close by and the QTL mapped to the location of the coactivator this may indicate either the level of coactivator expression or the allele expressed is important in mediating the changes that support lactation but would not give any information on the genes that NcoA2 acts upon. Further research into the importance of this gene to lactation is required.

Serum/glucocorticoid regulated kinase family, member 3 (SGK3)

This gene is one of the three members (SGK1, SGK2 and SGK3) of the serum and glucocorticoid-inducible family, a novel serine/threonine protein kinase family (Tessier and Woodgett 2006). SGK3 mRNA concentrations were similar in all human tissues tested, including heart, brain, liver and pancreas (Kobayashi *et al.* 1999a). SGK3 is activated by oxidation. Insulin and IGF-I activate SGK3 through a signalling cascade involving PI3-kinase, PDK1 and PDK2/H-motif kinase (Kobayashi *et al.* 1999a, Lang *et al.* 2006). The SGK3 protein has a role in the activation of potassium, chloride, sodium and calcium channels (Lang *et al.* 2006). Experiments using *Xenopus* oocytes injected with human, bovine or rat cRNA (cRNA is RNA derived from complementary DNA, which is DNA synthesized from mature messenger RNA) encoding SGK3 and various amino acid transporters, have shown that SGK3 up-regulates a variety of amino acid transporters (Boehmer *et al.* 2003, Boehmer *et al.* 2005, Bohmer *et al.* 2004, Palmada *et al.* 2005, Shojaiefard *et al.* 2005). SGK3 knockout mice (*sgk3*$^{-/-}$) showed decreased glucose transport across the intestine (Sandu *et al.* 2005). Experiments using *Xenopus* oocytes demonstrated a strong stimulatory effect of SGK3 on the Na^+-coupled glucose transporter 1 (SGLT1), known to be involved in intestinal and renal glucose transport (Dieter *et*

al. 2004). SGLT1 is present in the bovine mammary gland (Zhao *et al.* 1999). Although it is not the major glucose transporter present, SGLT1 mRNA levels were increased 4-fold ($P<0.01$) from 40 days before birth to 7 days after birth. It may have a role in substrate transport, possibly within the mammary gland, during lactation (Zhao *et al.* 2005, Zhao *et al.* 2004). In addition, SGLT is expressed along the gastrointestinal tract of dairy cows (Drackley *et al.* 2006, Zhao *et al.* 2005). The number and activity of these transporters were increased 60- and 90-fold, respectively, in response to glucose infusion, despite only a 2-fold increase in mRNA abundance (Drackley *et al.* 2006). Thus, post-translational modifications appear to be an important factor in the control of SGLT1 (Drackley *et al.* 2006). SGK3 may play an important role in increasing the activity of SGLT1, which would help to increase nutrient absorption across the gastrointestinal tract and increase nutrient availability for lactation.

Kinases, including SGK3, stimulate the voltage-gated K^+ channel complex KCNE1/KCNQ1 (Embark *et al.* 2003). The channels contribute to the maintenance of the electrical potential difference across the apical cell membrane, which is a critical driving force for glucose and amino acid transport (Rexhepaj *et al.* 2007). All three SGK isoforms stimulate the Na^+/K^+-ATPase, which is required for maintenance of the Na^+ gradient, an important driving force for Na^+-coupled nutrient transport (Henke *et al.* 2004). SGK3 may participate in the regulation of Na^+/K^+-ATPase activity through its activation by hormones such as insulin and IGF-1 (Henke *et al.* 2004).

SGK3 influences cell survival in a number of ways. SGK3 effectively blocks apoptosis induced by the withdrawal of the cytokine interleukin-3. In addition, SGK3 has been shown to up-regulate Kv1.3 (Kv channels are voltage-dependent (activated by depolarisation) potassium channels that are expressed in a wide range of cells and tissues) (Henke *et al.* 2004). IGF-I induced cell proliferation in human embryonic kidney cells (HEK293) and up-regulated Kv channels. PI3-kinase, PDK1 and SGK1 were shown to be involved in the IGF-I signalling pathway that caused this up-regulation (Gamper *et al.* 2002). The proliferative effect of IGF-I was disrupted by blockade of Kv channels, indicating a role for Kv channels in the proliferative

action of IGF-I (Gamper *et al.* 2002). Up-regulation of Kv1.3 channel activity, in part by the stimulation of SGK3, may be important for the proliferative effect of IGF-I (Henke *et al.* 2004).

Current knowledge of the SGK3 protein indicate that it has a role in the activation of ion channels (Lang *et al.* 2006), up-regulates a variety of amino acid transporters (Boehmer *et al.* 2003, Palmada *et al.* 2005, Shojaiefard *et al.* 2005) and has a stimulatory effect on SGLT1 (Dieter *et al.* 2004). Thus, a change in the expression of SGK3 may result in alteration in the activation of amino acid transporters and ion channels with a concomitant change in the drive to take up glucose and amino acids.

Differential regulation of proteins, such as changes in number or activity, occurs during early lactation, for example, the activity of the enzyme lipoprotein lipase is down-regulated in adipose tissue while it is up-regulated in the mammary gland during early lactation (Chilliard *et al.* 1977, McNamara *et al.* 1987, Shirley *et al.* 1973). Thus, it seems feasible that differential regulation of the SGK3 gene could occur during lactation and it may be regulated by changes in insulin and IGF-I concentrations in early lactation (Henke *et al.* 2004, Kobayashi *et al.* 1999a, Lang *et al.* 2006, Rhoads *et al.* 2004). Down-regulation of SGK3 may occur in tissues, such as skeletal muscle, that exhibit a reduction in utilisation of amino acids, glucose and acetate during lactation (Baracos *et al.* 1991, Pethick and Lindsay 1982, Vernon *et al.* 1990). The SGK3 gene may be up-regulated in tissues that must increase uptake of these substrates during lactation, such as the mammary gland and gastrointestinal tract (Annison 1983, Bickerstaff *et al.* 1974, Ha and Kennelly 1984, Okine *et al.* 1994).

Regulator of G-protein signalling 20 (RGS20/RGSZ1)

Regulator of G protein signalling (RGS) proteins are a family of GTPase-activating proteins (GAPs) for heterotrimeric guanine nucleotide binding proteins (G proteins). They share a conserved 120 amino acid RGS domain that binds directly to the Gα subunits to modulate G protein signalling (Hollinger and Hepler 2002). Extracellular signals, such as, light, hormones, neurotransmitters, and proteins activate 7-membrane-spanning G-protein coupled receptors

(GPCRs). These signals are then transmitted by G-proteins, to effector proteins inside the cell which initiates changes in cell behaviour (Abramow-Newerly *et al.* 2006, Glick *et al.* 1998, Nunn *et al.* 2006). Activation of GPCRs causes a conformational change that catalyses GDP (guanosine diphosphate) dissociation and GTP (guanosine-5'-triphosphate) binding on the Gα subunit, this leads to activation of its effectors and propagation of downstream signalling pathways (Nunn *et al.* 2006). GTP hydrolysis is accelerated by GAPs and terminates G protein activation (Wang *et al.* 1998). RGS proteins were initially identified as GAPs that increased the rate of hydrolysis of GTP-bound Gα subunit to inactivate it. Later it was found that they can regulate G protein-effector interactions in other ways that are not currently well understood (Abramow-Newerly *et al.* 2006, Nunn *et al.* 2006).

The RGS20 gene contains multiple introns, alternative splicing of this gene produces at least six mRNAs that encode protein variants that differ in size and sequence of their N-terminal domains. Therefore, they are likely to differ in their attachment to membranes and their subcellular distribution (Barker *et al.* 2001). These proteins include RGSZ1, Ret-RGS and four other proteins that have not yet been characterised (Barker *et al.* 2001). The expression of Ret-RGS proteins is limited to retinal interneurons (Barker *et al.* 2001). RGSZ1 was first isolated from bovine brain and subsequent studies have shown that RGSZ1 mRNA is expressed in multiple areas throughout the human brain (Barker *et al.* 2001, Wang *et al.* 1998). RGSZ1 protein exhibits high selectivity for $G\alpha_z$ and is able to increase the rate of GTP hydrolysis for $G\alpha_z$ up to 400-fold (Mao *et al.* 2004, Wang *et al.* 1998), it is strongly hydrophobic and firmly membrane-bound (Barker *et al.* 2001). $G\alpha_z$ hydrolyses bound GTP very slowly, suggesting that activated $G\alpha_z$ does not turn off immediately after receptor activation and implies that an efficient $G\alpha_z$ GAP is a required component of the $G\alpha_z$ signalling system (Nunn *et al.* 2006, Wang *et al.* 1998). Further studies suggest that RGSZ1 also interacts directly with members of $G\alpha_i$ subfamily and regulates $G\alpha_i$ mediated cell signalling (Mao *et al.* 2004, Wang *et al.* 2002) and lead to the suggestion that RGSZ1 is a selective $G\alpha_z$ GTPase-activating protein and a general inhibitor of signals mediated via members of the $G\alpha_i$ subfamily (Mao *et al.* 2004).

RGSZ1 blocked TRH-stimulated Ca^{2+} mobilisation in cultured cell lines either by acting as a GAP or by interacting with the G-protein to inhibit its interaction with the signalling effector (Mao *et al.* 2004). Studies in rat and mice pancreatic islet β- cells have demonstrated that $G\alpha_z$ mediated prostaglandin E inhibition of glucose-stimulated insulin secretion and RGSZ1 blocked the effects of $G\alpha_z$ (Kimple *et al.* 2005). A study in mice showed that RGSZ1 regulates the activity of opioids at μ but not δ receptors in the central nervous system (CNS) (Garzón *et al.* 2004).

As a result of the small amount of research that has been conducted on the RGS20 gene, it seems possible that the protein products of this gene may have a wide range of functions particularly considering there are six splice variants, which code for proteins of differing size and binding capabilities (Barker *et al.* 2001). Of particular note are the results that demonstrate an involvement of the RGSZ1 protein in regulating insulin secretion and the activity of opioids. Insulin secretion in response to insulinotropic substrates is reduced during lactation and is one of the many important adaptations in the physiological control of nutrient partitioning that contribute to redirecting nutrients towards the mammary gland during lactation (Holtenius *et al.* 2003, Lomax *et al.* 1979). RGSZ1 blocked $G\alpha_z$ mediated prostaglandin E inhibition of glucose-stimulated insulin secretion from rat and mice pancreatic islet β- cells (Kimple *et al.* 2005). RGSZ1 may be down-regulated during lactation, which would enable $G\alpha_z$ to reduce insulin secretion in response to insulinotropic agents. In addition, the RGSZ1 protein may also have a role in mediating opioid activity in the CNS (Garzón *et al.* 2004), opioids have a number of possible roles in physiological changes that occur during lactation (section 6.6), therefore this may be another way in which RGSZ1 plays a role in lactation. Currently there has been no research that has investigated whether RGSZ1, or any of the other RGS20 splice variants, have a role in the lactating animal. Thus, new research is required to investigate if this gene has a role in controlling any of the QTL in the chromosomal region of interest on BTA14.

RAB2, member RAS oncogene family (RAB2)

The product of the Rab2 gene is a member of the Rab family of small molecular weight, monomeric GTPases that interconvert between GDP- and GTP-bound states (Tisdale 1999). Rab proteins are required for vesicle targeting and fusion in both exocytic and endocytic pathways (Sallese *et al.* 2006). Rab2 is believed to modulate retrograde transport, which is transport from the Golgi to the endoplasmic reticulum (ER). Retrograde transport is essential for retrieval of ER or Golgi resident proteins. This protein retrieval is necessary to maintain organelle homeostasis and recycle components of the transport machinery required for anterograde, ER to Golgi, transport (Dong and Wu 2007, Tisdale *et al.* 1992).

The ER-Golgi intermediate complex (ERGIC) is a complex membrane system between the rough ER and the Golgi. It is the first post-ER sorting station for anterograde and retrograde protein traffic and thus plays a critical role in separating proteins for anterograde and retrograde transport in the early secretory pathway (Appenzeller-Herzog and Hauri 2006). Rab2 is localised to the ERGIC (Tisdale *et al.* 1992) and is thought to be a key player in intermediate compartment function (Tisdale 1999). It has been proposed to modulate retrograde protein transport specifically from the ERCIG to the ER by recruitment of soluble components necessary for protein sorting and recycling from pre-Golgi intermediates resulting in the generation of retrograde transported vesicles (Tisdale 1999).

Rab2 promotes Src membrane association to the ERGIC (Tisdale and Artalejo 2006). Src is a member of the non-receptor tyrosine kinase family that is expressed in most tissues and is involved in signal transduction events that regulate cell growth, survival, motility, differentiation, adhesion and migration (Thomas and Brugge 1997). Rab2 bound to ERCIG stimulates recruitment of atypical protein kinase C ι/λ (aPKCι/λ) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to ERGICs. Both of these proteins bind directly to Rab2 and also interact with each other (Tisdale 1999, 2003, Tisdale *et al.* 2004). Src mediated phosphorylation of atypical protein kinase C ι/λ (aPKCι/λ) is essential for interaction with Rab2-GAPDH-Src on the ERGIC (Tisdale and Artalejo 2006). aPKCι/λ promotes the binding

of COPI (coat protein complex 1, a protein complex crucially involved in transport vesicle assembly and intra-Golgi traffic) to intermediate compartment membranes, and phosphorylates GAPDH. GAPDH influences microtubule dynamics and transport in the early secretory pathway (Tisdale 2003, Tisdale and Artalejo 2006, Tisdale *et al.* 2004).

Recent studies have shown that Rab2-coordinated protein transport has an essential role in the anterograde transport and cell-surface targeting of α_{2B}-adrenergic receptor (AR) and β_2-AR (Dong and Wu 2007). These authors postulated that retrograde transport from the Golgi through the ERGIC to the ER may control the specificity of G protein-coupled receptors targeting to the plasma membrane (Dong and Wu 2007).

Transport of milk constituents through the mammary epithelial cell (MEC) can occur in a number of ways and is dependent on whether the constituent is synthesised within the MEC or is transported from plasma across the MEC into the lumen. There are three intracellular pathways that are represented diagrammatically in Figure 6.2 and are described below:

- In pathway I (Figure 6.2) milk constituents are synthesised in the MEC, transferred in vesicles, in which they are transported through the cell and expelled from the cell by exocytosis. Caseins, α-lactalbumin and other milk specific proteins are synthesised in the ER, which is extensive in the lactating MECs (Mather and Keenan 1998, Ollivier-Bousquet 2002). Lamellar cisternae of the ER, in close proximity to the cis side of the Golgi apparatus, form the ERGIC (Boisgard *et al.* 2001). The ERGIC plays a central role in the early part of the secretory pathway in the MEC (Péchoux *et al.* 2005). Vesicles then bud from the ER and fuse with the ERGIC at the cis-side of the Golgi apparatus (Mather and Keenan 1998, Ollivier-Bousquet 2002). These anterograde-directed vesicles are coated with a set of proteins termed COPII (Péchoux *et al.* 2005). COPII differs from COPI because it coats vesicles that are involved in anterograde transport whereas COPI-coated vesicles move bi-directionally between the ER and the Golgi (Péchoux *et al.* 2005). Maturation of the milk proteins, in particular caseins, may occur as they pass

through the Golgi apparatus. It has been suggested that proteins move across the Golgi stack as the cis cisternae mature to become medial and then trans cisternae (Boisgard *et al.* 2001). In addition, lactose is synthesised by the action of the galactosyltransferase α-lactalbumin complex inside the Golgi apparatus (Mather and Keenan 1998). On the trans side of the Golgi apparatus, budding of secretory vesicles occurs from the trans Golgi network (Boisgard *et al.* 2001). Secretory vesicles containing mature caseins, whey proteins, lactose, citrate and soluble salts release their contents into the lumen of the acinus by exocytosis (Boisgard *et al.* 2001, Ollivier-Bousquet 2002). More recent studies have suggested that tubular structures are involved in the transport of milk proteins in the secretory pathway (Péchoux *et al.* 2005). In addition, a small quantity of newly synthesised proteins, including milk proteins, hormones, receptors and enzymes are carried along a basolateral pathway to the basement/lateral membranes (Boisgard *et al.* 2001, Ollivier-Bousquet 2002).

- An endocytotic and transcytotic pathway (pathway 2, Figure 6.2) that involves internalisation of plasma-derived components into the cell and their transport into the milk. During transport, a sorting of internalised molecules strictly controls the destination of the transcytosed products (Ollivier-Bousquet 2002).

- Milk fat droplets (pathway 3, Figure 6.2) are carried from the ER where they are synthesised to the apical region; they then bud from the apical membrane and are released into the lumen encased in the mammary fat globule membrane (Ollivier-Bousquet 2002).

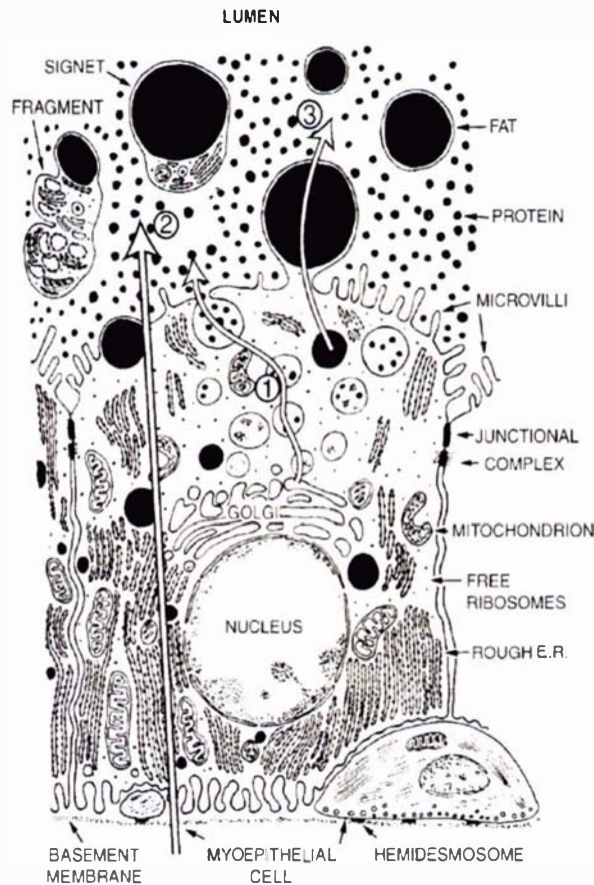


Figure 6.2 Diagram showing the major routes of secretion across the mammary epithelial cell. The pathways shown by arrows through the cell are: 1. pathways that ends with exocytosis; 2. endocytotic and transcytotic pathways; and 3. milk fat droplet pathway. Adapted from Linzell and Peaker (1971)

Members of the Rab family of small molecular weight, monomeric GTPases localise to specific subcellular compartments and are implicated at virtually all steps of membrane transport that have been studied (Gruenberg and Maxfield 1995). Rab2 bound to the ERGIC participates and stimulates interaction of a number of molecules resulting in binding of COPI to intermediate compartment membranes and phosphorylated GAPDH influences microtubule dynamics and transport in the early secretory pathway (Tisdale 2003, Tisdale and Artalejo 2006, Tisdale *et al.* 2004). COPI-coated vesicles mediate the anterograde transport from the ERGIC to the cis-most cisternae of the Golgi or the recycling from the Golgi and ERGIC to the ER (Péchoux *et al.* 2005).

It has been suggested that ER-to-Golgi trafficking could be perturbed if the recycling of molecules needed for the formation of anterograde vesicles was impaired (Péchoux *et al.* 2005).

It was observed in mammary epithelial cells from lactating rabbits that incubation with bromoenol lactone slowed down the transport of caseins from the ER to the Golgi stacks. The authors suggested that inhibition of retrograde transport of components involved in the forward transport of newly synthesised milk proteins to the Golgi complex could impair their transport (Péchoux *et al.* 2005). Thus, mechanisms that control vesicular traffic in both directions between the ER and the Golgi could be critical to the efficient and effective secretion of synthesised milk proteins from within the MEC. Since Rab2 is important for retrieval of ER or Golgi resident proteins (Dong and Wu 2007, Tisdale *et al.* 1992) it seems possible that this protein could have a role in the efficient functioning of MEC. If the ER and Golgi resident proteins and components of transport machinery are not recycled in a timely fashion this may reduce the efficiency of the secretory cells and reduce the speed at which proteins are transported from the ER to the Golgi apparatus. In addition, this pathway is particularly important in the secretion of milk proteins, which fits well with the QTL found in the region of interest in this study. If Rab2 was the gene underlying the 60 day milk QTL, it is unclear why it would only affect these parameters in early lactation. Nevertheless, in view of the huge amount of vesicular movement in the secretory cell it would be unwise to ignore the possibility that this gene is involved.

ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited) (ARFGEF1)

The ADP-ribosylation factor guanine nucleotide-exchange factor 1 (brefeldin A-inhibited) (ARFGEF1) gene product was first isolated from bovine brain cell cytosol and is a ~200-kDa molecule which is also known as BIG1 (for brefeldin A-inhibited guanine nucleotide-exchange protein) (Morinaga *et al.* 1996, Togawa *et al.* 1999). ARFGEF1 was primarily located in the cytosol and associated with the Golgi in cells growing in culture medium (Padilla *et al.* 2004). A large percentage of these proteins were found in the nuclei when these cells were incubated without serum (Padilla *et al.* 2004). As a result the authors suggested that ARFGEF1 participates in both nuclear and Golgi systems. It may be a signalling and scaffolding protein for

integration of alterations that both of these organelles must undergo throughout the cell cycle (Padilla *et al.* 2004).

The ARFGEF1 protein has been reported to preferentially activate ADP-ribosylation factors (ARFs) 1 and 3. ARFs have essential regulatory roles in the formation of membrane trafficking vesicles in eukaryotic cells. They exist in the cytosol in a GDP-bound inactive state. Conversion to a GTP-bound active form, that is tightly membrane-associated, is accelerated by guanine nucleotide-exchange factors (GEFs) such as ARFGEF1 (Citterio *et al.* 2006, Padilla *et al.* 2004). ARF1 and ARF3 mediate ER-Golgi trafficking and thus are involved in the early protein secretory pathway. ARF1 activation has been shown to stimulate the assembly of spectrin and the actin cytoskeleton on Golgi, this is important for integrity of Golgi structure and formation and/or dissociation of newly formed vesicles (D'Souza-Schorey and Chavrier 2006, Padilla *et al.* 2004, Shen *et al.* 2007). ARF1 is the master regulator of many fundamental steps along the secretory pathway, including assembly of coat proteins such as COPI and clathrin (Sallese *et al.* 2006). Thus, ARFGEF1 action in the late Golgi region causes protein trafficking by activating ARF1 or 3, which in turn initiate the generation of vesicles for transport to the cell surface (Shen *et al.* 2007). ARFGEF1 is also believed to play a role in cAMP regulation of Golgi structure (Kuroda *et al.* 2007).

The three intracellular transport pathways in the MEC were briefly described on page 161-162. Milk proteins and lactose are transported along pathways that end in exocytosis. Secretory vesicles bud from the trans Golgi network and contain mature caseins, whey proteins, lactose, citrate and soluble salts that are then released into the lumen of the acinus by exocytosis (Boisgard *et al.* 2001, Ollivier-Bousquet 2002). Movement of vesicles from the Golgi to the apex of the cell could be a critical step in the process of secretion of these milk constituents. The involvement of the ARFGEF1 protein product in the initiation of vesicle budding from the late Golgi region could be a rate-limiting control point in the secretory pathways of milk proteins and lactose.

The very high levels of milk production achieved by some cows may be dependent on the ability of the MECs to secrete milk proteins and lactose. ARFGEF1 may play a role in the timing and regularity of vesicle formation. Differences between animals may result from differences in gene expression, different alleles or post-translational modifications. Despite not knowing the full extent of the actions of the ARFGEF1 protein, it can not be discounted as a possible candidate gene for the early lactation milk production QTL found in the region of interest on BTA 14. Perhaps the secretory pathways are under the greatest load during early lactation when milk production is increasing to a peak and it is during this time that differences between animals in the control of secretory pathways has consequential effects on their milk and protein yields.

ATPase, H+ transporting, lysosomal 50/57kDa, V1 subunit H (ATP6V1H)

The ATP6V1H gene encodes the H subunit of the V_1 sector of the vacuolar H^+-ATPase (V-ATPase). The V-ATPase is a multi-subunit enzyme that is divided into two distinct domains or sectors (Figure 6.3). V-ATPase is thought to be expressed in virtually all eukaryotic cells and is involved in the translocation of protons across the membrane of various intracellular organelles, resulting in their acidification (Beyenbach and Wieczorek 2006, Breton and Brown 2007, Morel 2003).

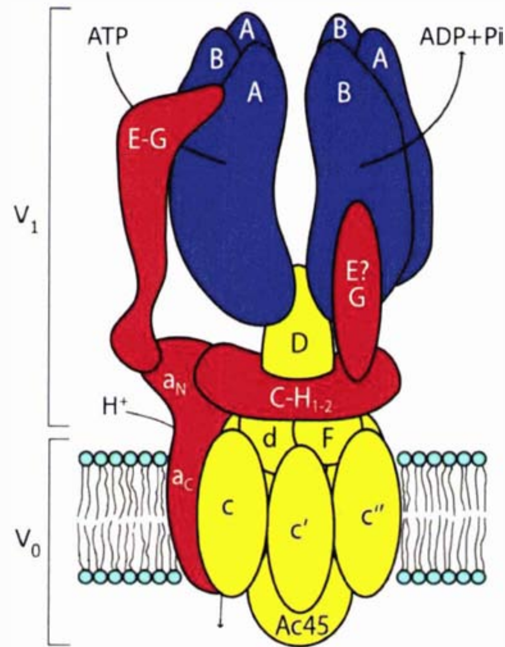


Figure 6.3 A diagrammatic representation of the bovine V-ATPase adapted from: Gregorini *et al.* 2007, Sun-Wada *et al.* 2004, Wilkens *et al.* 2004). The transmembrane V_0 sector is responsible for proton translocation and is composed of 6 subunits (subunits designated by lower case letters), while the V_1 sector forms a large cytosolic complex composed of 8 subunits (designated A-H) and carries out ATP hydrolysis and nucleotide binding (Inoue *et al.* 2005, Nishi and Forgac 2002, Xu *et al.* 1999). The V-ATPase uses the energy from ATP hydrolysis to drive proton transport across membranes (Sun-Wada *et al.* 2004). Three copies of the A subunit alternate with three copies of the B subunit to form the part of the V_1 sector, which is connected to the V_0 sector via two or more stalks, composed of subunits C to H. (Breton and Brown 2007, Xu *et al.* 1999).

Three alternatively spliced transcript variants encode two isoforms of the H subunit, both of which are present in the stalk of the V-ATPase enzyme, connecting the V_0 and V_1 sectors (Lu *et al.* 2002, Wilkens *et al.* 2004). Studies in yeast have shown that the V-ATPase enzyme is still assembled in cells that are devoid of subunit H but it is less stable and more importantly, the enzyme is inactive (Ho *et al.* 1993). Studies in bovine cells demonstrated that loss of subunit H results in loss of activity in V-ATPase (Xu *et al.* 1999). V-ATPase is involved in endocytosis mediated by clathrin-coated pits, and is required for the formation of endosomes. Subunit H interacts with the AP-2 adaptor protein involved in clathrin-mediated endocytosis (Breton and Brown 2007).

V-ATPase is responsible for providing most of the energy required for transport processes in the vacuolar system. V-ATPase dependent organelle acidification is necessary for intracellular processes such as protein processing and degradation, zymogen activation, receptor-mediated

endocytosis, synaptic vesicle proton gradient generation, and the coupled transport of small molecules such as neurotransmitters (Inoue *et al.* 2005, Morel 2003). In exocytotic pathways, peptides and proteins in acidic secretory vesicles are often processed from pro-proteins by acid proteases or enzymes to yield, for example, enkephalin or insulin (Beyenbach and Wieczorek 2006, Sun-Wada *et al.* 2006).

Mice with a subunit of the V-ATPase deleted had significantly lower plasma insulin concentrations, decreased islet insulin content and decreased insulin secretion compared with wild-type mice; leading to the suggestion that this deletion caused the exocytosis step of secretory granules to be defective (Sun-Wada *et al.* 2006). In addition to its likely role in the pancreas, V-ATPase may be important in other endocrine organs. Subunits of V-ATPase are expressed in the secretory granules in organs including the adrenal gland, parathyroid gland, pituitary gland and pineal gland, all of which have important endocrine functions in maintenance of whole body homeostasis (Sun-Wada *et al.* 2006). The presence of V-ATPase subunits in the secretory granules of many endocrine organs may indicate that this enzyme has a role in hormone secretion.

The period of negative energy and protein balance in early lactation is accompanied by changes in circulating concentrations of important homeorhetic hormones, including decreased insulin and IGF-I and increased GH plasma concentrations (Accorsi *et al.* 2005, Bell *et al.* 2000, Gulay *et al.* 2004, Holtenius *et al.* 2003, Ingvartsen and Andersen 2000, Komatsu *et al.* 2005, Lacasse *et al.* 1994, Rhoads *et al.* 2004, Spicer *et al.* 1990, Vega *et al.* 1991). The changes in hormone concentrations at the onset of copious milk synthesis and secretion affect the partitioning of nutrients between milk production in the mammary gland and the needs of non-mammary tissues (Bauman and Currie 1980, Chilliard 1999). Cows that differ in genetic merit and measured milk production have different plasma hormone concentrations, for example IGF-I, insulin and GH (Bonczek *et al.* 1988, Hart *et al.* 1980, Kazmer *et al.* 1986, Knight *et al.* 2004, Snijders *et al.* 2001).

It is possible that changes in gene transcription, translation or post-translational modifications of V-ATPase in the secretory granules of endocrine glands would alter the secretion of the hormone it is carrying. Hormone secretion may either be increased or decreased depending on the changes to V-ATPase. Thus, changes in circulating hormone concentrations in early lactation may be a result of changes in secretion due to alterations in V-ATPase. The H subunit of the V-ATPase, encoded by ATP6V1H, is a good candidate, particularly for decreasing hormone secretion, because loss of subunit H from bovine cells results in loss of activity in the V-ATPase (Xu *et al.* 1999).

V-ATPase in the plasma membrane functions in renal acidification, bone resorption, tumour metastasis, sperm motility and maturation and maintenance of pH in the inner ear (Nishi and Forgac 2002, Xu *et al.* 1999). V-ATPase enzymes are expressed and functionally active in ovine rumen epithelial cells and it has been suggested that they play a role in intracellular pH regulation and establishment of transport gradients (Etschmann *et al.* 2006). The increase in nutrient demands brought about by the onset of lactation is accompanied by an increase in food intake and an increase in the absorptive capacity of the gastrointestinal tract, as a result of greater mass, greater enzyme activities and greater transport capability (Fell *et al.* 1972, Ha and Kennelly 1984, Okine *et al.* 1994, Reynolds *et al.* 2004, Sutter and Beever 2000, Weekes 1972). It is possible the increase in absorptive capacity has a crucial role in increasing nutrient uptake in early lactation dairy cows. Differences may exist between cows in their ability to increase absorptive capacity of the gastrointestinal tract and V-ATPase may play a role in this. Perhaps some alleles for subunits of the V-ATPase enzyme confer greater efficiency to the enzyme than others so cows that possess the alleles with greater efficiency would be able to increase the absorptive capacity of the gastrointestinal tract more effectively than others.

However, the weakness with both of these hypotheses is that there no milk fat yield QTL in the region of the chromosome under consideration. It could be argued that an increase in supply of nutrients to the mammary gland, either from greater nutrient partitioning towards the mammary gland or increased absorptive capacity in the gastrointestinal tract, would result in changes in all

components of milk not just proteins and lactose. However, some studies have indicated that the supply of amino acids are limiting to milk production (Kim *et al.* 2000, Kokkonen *et al.* 2002, Mackle *et al.* 1999). If the hypothesised actions of V-ATPase in the lactating animal increased the availability of amino acids then perhaps it would influence lactose and milk protein yield more so than fat yield. Further knowledge of the function of this enzyme, in particular in relation to lactation, is needed before a firm conclusion can be drawn.

Summary

The functions of six genes were discussed in the section. Genes with a function that could be logically considered to be involved in any one of the vast number of changes in metabolic and secretory pathways that support lactation were investigated. Nuclear receptor coactivator 2 is a transcriptional coactivator that has an important role in energy homeostasis and possibly mammogenesis during pregnancy. The serum/glucocorticoid regulated kinase family, member 3 protein has a role in the activation of ion channels, up-regulates a variety of amino acid transporters, has a stimulatory effect on SGLT1 and may in part be regulated by insulin and IGF-I. NcoA2 and SGK3 were considered to be the most suitable candidates because the protein products of these genes could be involved in key regulatory steps in a number of pathways that are likely to be relevant to milk production in the first 60 days of lactation.

6.6 Food intake control candidate genes

Loss of live weight in early-lactation, high-producing dairy cows has been reported in numerous studies. This reflects a general inability of high-producing dairy cow to sufficiently increase post-partum feed intake to meet energy demands caused by the rapid increase in milk production (Clark *et al.* 2005, Gulay *et al.* 2004, Roche *et al.* 2006a, Spicer *et al.* 1990, Yan *et al.* 2006). This is particularly so in the case of pasture fed cows (Clark *et al.* 2005) due to pasture diets not being as ideally suited to the requirements of milk production as total mixed ration diets ((Burke *et al.* 2002, Waghorn 2002). A number of experiments have demonstrated that high genetic merit cows produced more milk and have a greater DMI than medium or low genetic merit cows (Kennedy *et al.* 2003, Linnane *et al.* 2004, Snijders *et al.* 2001, Yan *et al.*

2006). In an experiment studying dairy cows in the first 26 weeks of lactation, milk yield was positively correlated with feed intake, live weight immediately post-calving, and live weight loss over the lactation period (Neilson *et al.* 1983). In addition, positive genetic (0.28) and phenotypic (0.34) correlations have been reported between DMI and milk yield (Veerkamp *et al.* 2000). Consequently, the ability of a dairy cow to increase intake is an important factor in achieving greater milk yield, particularly in early lactation. Therefore, genes involved in controlling food intake are discussed in this section.

The genes that will be discussed in this section are:

- Opioid receptor, kappa 1 (OPRK1),
- Proenkephalin (PENK),
- Corticotropin releasing hormone (CRH) and
- Neuropeptides B/W receptor 1 (NPBWR1).

These genes have been included in this section because one of their functions is an involvement in an aspect of the control of food intake. Thus, the currently known functions and the possible role each gene may play in influencing the QTL in the region of interest in this investigation will be discussed in this section.

Opioid receptor, kappa 1 (OPRK1)

Opioid receptor, kappa 1 (OPRK1, also known as kappa opioid receptor-1(KOR-1)) is the κ1 subtype of one of four known types of opioid receptors (μ, δ, κ and nociception) (Bodnar 2004). Opioid receptors are a class of receptors that belong to the large super family of GPCRs (Waldhoer *et al.* 2004). Opioid receptors occur in a variety of hypothalamic and pituitary sites (Kim *et al.* 1997). They are bound/activated by opioid peptides with subsequent activation of G proteins that in turn alter membrane conductances of K^+ and Ca^+ and alter levels of second messengers such as cAMP and inositol 1,4,5-trisphophate (IP_3) (Uhl *et al.* 1994). OPRK1 may

play a role in arousal and regulation of autonomic and neuroendocrine functions, such as immune response, signal transduction, G-protein coupled receptor protein signalling, adenylate cyclase inhibiting pathway, synaptic transmission, sensory perception and behaviour (Chefer *et al.* 2005).

Systemic administration of κ-agonists has been shown to increase food intake in rodents, rabbits, pigs and humans (Baldwin *et al.* 1990, Bodnar 2004, Jackson and Cooper 1985, Sipols *et al.* 2002). Blockade of opioid receptors, by ventricular administration of κ-antagonists, decreased food intake induced by a number stimuli including neuropeptide Y (NPY) and agouti-related protein (AgRP), glucose deprivation, food deprivation and offering a high fat diet (Arjune and Bodnar 1990, Barton *et al.* 1996, Bodnar 2004, Brugman *et al.* 2002, Israel *et al.* 2005, Kotz *et al.* 1993, Levine *et al.* 1990, Silva *et al.* 2002b). In addition, intracerebroventricular (ICV) administration of insulin in rats abolished the doubling of feed intake caused by κ-opioid receptor agonist administration (Sipols *et al.* 2002). This evidence suggests that the κ-opioid receptor is involved in mediation of food intake and possibly involved in increased food intake associated with energy needs.

Treatment of lactating rats with opioid antagonists resulted in a small reduction in spontaneous nocturnal food intake in one study and no change in feed intake in another study compared with controls (Kim *et al.* 1997, Wager-Srdar *et al.* 1985). OPRK1 is a receptor for dynorphin, a type of opioid peptide. mRNA concentrations of prodynorphin were significantly lower in the arcuate nucleus (ARC) of lactating rats compared with non-lactating control rats (Kim *et al.* 1997). The large energy demands experienced due to the onset copious milk synthesis and secretion was likened to the animal being chronically food restricted (Kim *et al.* 1997). Evidence for this is shown by the significantly higher NPY concentrations in the paraventricular nucleus (PVN) of rats subjected to a 48 hour fast, or the ARC in lactating rats, compared with control rats (Kotz *et al.* 1997, Lambert *et al.* 1994, Smith 1993). In addition, the lower prodynorphin concentrations during lactation were consistent with a previous study in which food intake was either chronically restricted by 10 to 40% or when food deprivation was

imposed for 48 hours resulting in significantly decreased prodynorphin mRNA concentrations in the ARC (Kim *et al.* 1996).

Conversely, other studies have shown prodynorphin mRNA and dynorphin increase in certain brain regions in response to lactation and food restriction. A study in rats showed the mRNA concentrations of prodynorphin in the PVN and the supraoptic nucleus in the hypothalamus increased during lactation compared with non-lactating rats (Lightman and Young 1987). Chronic food restriction in rats produced an increase in dynorphin A_{1-17} in three regions, all components of the medial hypothalamus and increased prodynorphin mRNA concentrations in the central amygdale and the lateral hypothalamus (Berman *et al.* 1994, Berman *et al.* 1997). Furthermore, injection of dynorphin into the ventral medial hypothalamus increased food intake in non-food-deprived rats (Gosnell *et al.* 1986). The effect of medial hypothalamic dynorphin is likely to be mediated by κ-receptors as this region of the brain has dense κ-receptor binding and virtually no μ- or δ-receptor binding (Mansour *et al.* 1987). Further evidence for the involvement of κ-receptors was provided in an experiment in which consumption of a high-fat diet was reduced, compared with controls, in rats fasted for 20 hours when a κ-receptor antagonist was injected into the lateral cerebral ventricle immediately prior to the introduction of food (Barton *et al.* 1996).

Thus, opioid peptide and mRNA response to food deprivation and lactation are highly variable. This variability is influenced by the brain site examined, duration of feed restriction, time of sampling (due to strong circadian rhythms), and time since last meal (Kim *et al.* 1996). Although the aforementioned results are inconsistent, there is a considerable body of evidence indicating that opioid peptides and their receptors are involved in the control of food intake. In particular, dynorphin peptide and mRNA concentrations change in response to food restriction and lactation. Since κ-receptors have been shown to be the primary receptor mediating the effects of dynorphin on feed intake (Silva *et al.* 2002a, Silva *et al.* 2002b) it is possible that expression of the OPRK1 gene is important in the control of food intake. Given that opioids and their receptors are likely to influence food intake, it seems possible that the increase in food

intake in dairy cows, which occurs steadily over early lactation (Bauman and Currie 1980, Ingvartsen and Andersen 2000, Moussavi *et al.* 2007, Sutter and Beever 2000), may involve the opioid system. Thus, OPRK1 may have an effect on the first 60 days milk production and baseline IGF-I QTL through its affects on food intake.

One further consideration for a role of OPRK1 in early lactation is its role in regulating prolactin secretion. Agonist activation of κ-receptor stimulates prolactin secretion (Krulich *et al.* 1986). In addition, administration of κ- and μ-receptor antagonists prevent the nocturnal plasma prolactin surge that occurs during early pregnancy, the ante-partum prolactin surge and the prolactin surge that occurs in response to suckling during lactation (Andrews and Grattan 2003, Baumann and Rabii 1991). It has been suggested that prolactin secretion during pregnancy and lactation is enhanced by opioid-mediated inhibition of tuberoinfundibular dopamine (TIDA) neurones (TIDA neurons are the predominant source of dopamine which acts to inhibit prolactin secretion) acting at the κ- and μ-receptors (Andrews and Grattan 2003). This may be important with respect to the 60 day milk production QTL in this region. Cows treated with 2-Br-α-ergokryptin during the periparturient period had reduced basal serum prolactin and the normal prolactin surges that occur at parturition and milking were blocked resulting in a considerable reduction in milk production during the first 10 days of lactation (Akers *et al.* 1981). In addition, prolactin promotes milk protein gene expression including the casein gene in mammary tissue (Yang *et al.* 2000), and is likely to be involved in mammary cell proliferation (Akers 2006). Thus, OPRK1 may play a role in lactation through increasing food intake and regulating prolactin secretion.

Proenkephalin (PENK)

Proenkephalin belongs to the opioid neuropeptide precursor family. Proenkephalin is the precursor peptide of enkephalins, a type of opioid peptide, and contains four copies of methionine-enkephalin (met-enkephalin) and single copies of leucine-enkephalin (leu-enkephalin), ME-Arg-Gly-Leu (ME-RGL) and ME-Arg-Phe (Noda *et al.* 1982). Met- and leu-enkephalins play a role in a number of physiological functions, including pain perception and

responses to stress, however only those which were considered relevant to the QTL in the region of interest are included here. Enkephalins act predominantly at μ- and δ-opioid receptors (Clarke *et al.* 2003, Krulich *et al.* 1986). Enkephalin-containing neurons are located in many regions of the brain including a number of nuclei in the hypothalamus (Baile *et al.* 1987, Zamir *et al.* 1985).

A number of experiments have investigated a possible role for PENK in the regulation of food intake. In one experiment chronically restricted food intake in rats caused decreased PENK mRNA levels in the ARC in comparison with the levels in *ad libitum* fed controls and there was a correlation between body weight change and mRNA levels of PENK ($r = 0.455$, $P = 0.0028$). (Kim *et al.* 1996). However, food deprivation for 48 hours did not affect PENK mRNA expression (Kim *et al.* 1996). Administration of a δ-opioid receptor antagonist into the lateral ventricle of the brain blocked the anticipated increase in food intake following treatment with a free fatty acid oxidation inhibitor (Stein *et al.* 2000), thus supports the findings of Kim *et al.* (1996). In contrast, PENK mRNA tended to be down-regulated in the striatum of rats that had recently consumed a meal and up-regulated in rats that were denied food during their normal feeding period (Will *et al.* 2007). This pattern was observed regardless of whether the rats had been kept on a chronic food restricted diet or not, leading these authors to suggest that PENK may be involved in immediate aspects of food consumption and short-term satiety (Will *et al.* 2007). Food intake was inhibited by ICV injection of leu-enkephalin in sheep leading to the suggestion that δ agonists suppress feed intake (Baile *et al.* 1987). In addition, insulin deficiency caused by alloxan injection resulted in decreased met-enkephalin in both the anterior and neuro-intermediate lobes of the pituitary (Tang 1991), which may suggest that PENK has some involvement in energy homeostasis.

Enkephalins were decreased in the paraventricular (PVN) and ventromedial (VMH) nuclei of the hypothalamus in chronically food-restricted sheep. The VMH is important in the central satiety response and regulation of energy expenditure, thus alterations in PENK expression in the VMH may be directly involved in appetite and energy homeostasis (Henry *et al.* 2000).

Two experiments were conducted in adult Corriedale ewes, using the same animal treatment protocol, in which one treatment group were fed to maintain a body weight of 60 kg (FAT) and the other to maintain a body weight of around 38 kg (LEAN) (Henry *et al.* 2000, Iqbal *et al.* 2005). In one of these experiments enkephalin expression was increased in the periventricular nucleus (PeV) of the hypothalamus in LEAN compared with FAT treatment ewes. In the other experiment somatostatin immunoreactive cells immunostained for PENK, which indicated co-expression between PENK and somatostatin (Henry *et al.* 2000, Iqbal *et al.* 2005). Although the relationship is not yet clear, the co-expression of PENK and somatostatin in the PeV is suggestive of an association between these peptides. Thus, PENK may play a role in the control of GH secretion (Iqbal *et al.* 2005), which shows a dramatic increase in circulating concentrations in dairy cows in early lactation (Ronge *et al.* 1988, Vicini *et al.* 1991).

A number of experiments have demonstrated that PENK mRNA expression increased, in certain regions of the hypothalamus, preoptic regions and nucleus accumbens, in lactating compared with non-lactating rats, mice and sheep (Broad *et al.* 1993, Gammie *et al.* 2005, Ottinger *et al.* 1995, White and McKelvy 1986, Xiao *et al.* 2005). Morphine interacts preferentially with μ-opioid receptors and has been shown to stimulate release of prolactin and growth hormone (Krulich *et al.* 1986). Thus, the increase in PENK mRNA expression in lactating animals and the resulting increase in enkephalin neuropeptides may be involved in the regulation of prolactin and growth hormone, which fits well with the co-localisation of PENK and somatostatin immunoreactive cells in the PeV, discussed previously (Ottinger *et al.* 1995, White and McKelvy 1986). Contradictory to other studies, Kim *et al.* (1997) reported that mRNA levels of PENK were lower in the ARC of lactating rats than in post-lactating rats.

An experiment in lactating rats demonstrated that prolactin provided the primary regulatory influence for the suckling-induced increase in proenkephalin gene expression specific to the hypothalamic nuclei (Nahi and Arbogast 2003), while blockade of μ- and κ-opioid receptors prevented prolactin secretion in response to suckling (Andrews and Grattan 2003, Baumann and Rabii 1991). Another experiment in rats showed that during lactation there is a significant up-

regulation of enkephalin immunoreactivity and mRNA in the TIDA neurons (page 174) compared with male rats and cycling female rats (Merchenthaler 1993). The increased enkephalin in the TIDA neurons in lactating animals may cause the reduced activity of TIDA neurons observed during lactation (Andrews and Grattan 2003, Merchenthaler 1993, Xiao *et al.* 2005), and the concomitant decrease in dopamine activity with suckling-induced prolactin secretion.

The exact role of the opioid system in the control of food intake remains uncertain. An experiment in sheep demonstrated that regions of the hypothalamus vary in their enkephalin response to underfeeding. Enkephalin increased in some regions and decreased in others in response to the animals being maintained at a live weight 40% less than controls (Henry *et al.* 2000). This may explain why some experiments find increases and others find decreases in PENK in various regions of the brain in response to changes in food intake and as a result of lactation. In addition, experiments may not be comparable if the lactating animals are in different energy balance or on different planes of nutrition. Given the large number of different regions in the brain that PENK and its opioid receptors are expressed in, it is likely their role encompasses a number of aspects of ingestion behaviour and interactions with hypothalamic peptides, thus the opioid system may play a role in long-term regulation of energy balance (Brugman *et al.* 2002).

There are several potential roles for PENK in lactation. As already mentioned, PENK appears to be involved in prolactin and GH release (Broad *et al.* 1993, Henry *et al.* 2000, Iqbal *et al.* 2005, Nahi and Arbogast 2003, Ottinger *et al.* 1995, White and McKelvy 1986). The importance of prolactin during lactation was discussed on page 174. In high-producing dairy cows blood concentrations of GH increase dramatically around the time of onset of lactation (Accorsi *et al.* 2005, Kobayashi *et al.* 1999b, Kunz *et al.* 1985). The increase in circulating GH concentration plays an important role in partitioning nutrients towards the mammary gland by increasing lipolysis, decreasing lipogenesis, increasing gluconeogenesis, impairing the actions of insulin and increasing cardiac output and blood flow to the mammary gland (Borland *et al.*

1994, Davis *et al.* 1988, Etherton and Bauman 1998, Houseknecht and Bauman 1997, Knapp *et al.* 1992, Rose *et al.* 1996, Sechen *et al.* 1990, Vernon 1982, Vernon and Finley 1988, Watt *et al.* 1991). Dairy cows that have been selected for high milk production, and have a greater measured milk production, have higher plasma GH concentrations than unselected or control animals (Barnes *et al.* 1985, Kazmer *et al.* 1986, Lukes *et al.* 1989, Roche *et al.* 2006b, Sartin *et al.* 1988, Weber *et al.* 2007). Associations between milk yield and plasma concentrations of GH, both between cows and throughout lactation, indicates a lactotrophic effect of this hormone in cattle (Hart *et al.* 1978, Løvendahl *et al.* 1991a).

Food intake increases progressively for the first 8-12 weeks of lactation in the dairy cow (Bauman and Currie 1980, Kokkonen *et al.* 2002, Kunz *et al.* 1985, Ordway *et al.* 2002). In general, increase in food intake is relatively slow in relation to the increase in observed milk yield (Bauman and Currie 1980, Friggens *et al.* 1998). Adaptation in feed intake is an important component in the regulation of nutrient partitioning occurring in an integrated manner with the alterations in other processes during early lactation (Bauman *et al.* 1985). The rate and extent of increase in feed intake is highly variable between lactating cows (Ingvartsen and Andersen 2000). Experiments have found that dry matter intake is greater in cows with high genetic merit for milk production and higher actual milk production compared with low or medium genetic merit cows (Bryant and Trigg 1981, Kennedy *et al.* 2003, Roche *et al.* 2006b, Yan *et al.* 2006). Consequently, the action of PENK in the control of food intake may be important in relation to lactation. Differences in the ability of animals to increase food intake at the onset of lactation may be a factor influencing differences between animals in their ability to produce milk.

PENK may be an excellent candidate gene for the QTL in the region of interest due to its possible functions in both control of food intake and secretion of hormones that are important to lactation. This gene may play a key role in co-ordinating the physiological changes that must occur at the onset of lactation.

Corticotropin releasing hormone (CRH)

Corticotropin-releasing hormone (CRH) is a 41-amino acid peptide and was first isolated from ovine hypothalamic extracts in 1981 (Vale *et al.* 1981) and is synthesised in the parvocellular neurons of the paraventricular nucleus (PVN) of the hypothalamus (Carroll *et al.* 2007). It is an important ACTH secretagogue and is the major hypothalamic activator of the hypothalamo-pituitary-adrenal (HPA) axis during basal and stressful conditions (Mastorakos and Zapanti 2004, Watts 2005, White 2006).

CRH is secreted from the CRH neuroendocrine terminals in the median eminence and travel via the hypophysial portal vasculature to the pituitary. In the anterior pituitary it binds to the type 1 CRH receptor on the corticotrophs and stimulates adenylate cyclase activity increasing cAMP levels (Carroll *et al.* 2007, Majzoub 2006, Watts 2005). The result is the secretion of preformed ACTH and other pro-opiomelanocortin (POMC)-derived peptides while simultaneously stimulating POMC gene transcription, peptide synthesis and post-translational processing (Mastorakos and Zapanti 2004, White 2006). ACTH stimulates the adrenal gland to synthesise and secrete progesterone, androgen and glucocorticoids (Carroll *et al.* 2007). Glucocorticoids exert diverse affects on target tissues to mobilise energy for the body to deal with the stressor (Yao and Denver 2007).

CRH mRNA and the peptide synthesised from it are widely expressed throughout the CNS (in the hypothalamus and other regions) and in peripheral tissues, such as the adrenal gland, spleen heart and placenta, suggesting that CRH has wide ranging actions (Richard *et al.* 2002, Yao and Denver 2007). CRH has a number of other neuroendocrine actions, including the modulation of GnRH secretion in the hypothalamus to suppress reproductive hormone secretion (Calogero *et al.* 2002), and it is also involved in induction of inflammatory response and other aspects of the immune system (Zoumakis *et al.* 2006).

An acute infusion of CRH into the third ventricle of rats or an ICV infusion into sheep caused a decrease in food intake (Arase *et al.* 1988, Ruckebusch and Malbert 1986). Chronic CRH

infusion caused a prolonged and steady decline in body weight, while thermogenesis was increased under both treatment regimes (Arase *et al.* 1988). CRH gene expression and peptide synthesis in the rat hypothalamus were increased by ICV administration of leptin. Leptin is predominantly secreted by white adipose tissue, circulating concentrations are positively correlated with percent body fat (Rosen and Spiegelman 2006, Trayhurn and Bing 2006). Increased plasma leptin in response to food intake or increased adiposity is a key signal that decreases food intake and increases energy expenditure by acting on specific receptors in the hypothalamus (Okamoto *et al.* 2001, Rosen and Spiegelman 2006). Leptin action to decrease food intake was partially blocked by either CRH receptor anatagonists or an anti-CRH antibody (Gardner *et al.* 1998, Gotoh *et al.* 2005, Okamoto *et al.* 2001, Uehara *et al.* 1998). ICV administration of a melanocortin receptor agonist, into freely moving rats, increased CRH mRNA expression in the PVN and the suppressive effect of the melanocortin receptor agonist on food intake was impaired by a CRH receptor antatgonist (Lu *et al.* 2003). These findings suggest that CRH in the PVN is the downstream mediator of both leptin and melanocortin signalling (Shimizu *et al.* 2007). It has also been postulated that prolactin-releasing peptide may elicit anorectic effects partly through CRH neurons (Lawrence *et al.* 2004).

A few studies have investigated the role of the HPA axis in lactation in the dairy cow. One study found that a high level of milk production and the concomitant negative energy balance were associated with greater pituitary (re)activity (increased baseline ACTH concentrations and response to CRH) and decreased adrenocortical reactivity (cortisol responses to ACTH) compared with cows that were low producers (Beerda *et al.* 2004). Baseline plasma ACTH concentrations were significantly higher in early lactation than in the 2 weeks before calving (Beerda *et al.* 2004). The CRH-induced ACTH response was negatively correlated with plasma glucose concentrations (Beerda *et al.* 2004). Another experiment in dairy cows showed that milk, non-fat solids and milk fat solids yields were higher in cows being fed 5 or 10 μg of a synthetic glucocorticoid than animals fed 0 or 20 μg of a synthetic glucocorticoid (Swanson and Lind 1976). This suggests there is an optimal range of endogenous corticosteroid production

that will assist in maximising lactation potential (Holmes *et al.* 2002). Corticosteroids may affect the availability of nutrients to the mammary gland, they drive protein degradation, decrease protein synthesis and inhibit glucose uptake by skeletal muscle in addition to promoting hepatic gluconeogenesis (Vegiopoulos and Herzig 2007). However, experiments in lactating rats have demonstrated a possible uncoupling of the HPA axis from adrenocortical activity, that is, ACTH does not appear to be responsible for the high circulating concentrations of cortisol as both ACTH concentration and CRH mRNA were low during lactation (Fischer *et al.* 1995). It is uncertain if CRH, in its role as the major activator of the HPA-axis, has an influence on the control of lactation.

Plasma concentrations of leptin in dairy cows were reduced around the time of parturition and coincided with increased use of endogenous lipids as an energy source but occurred prior to significant depletion of white adipose tissue (Block *et al.* 2001, Leury *et al.* 2003). The onset of negative energy balance appears to be partly responsible for the lower concentration of plasma leptin in early lactating dairy cows (Block *et al.* 2001). As mentioned previously, centrally administered leptin causes an increase in CRH, while CRH receptor antagonists partially block the action of leptin in decreasing food intake (Gardner *et al.* 1998, Gotoh *et al.* 2005, Okamoto *et al.* 2001, Uehara *et al.* 1998). In addition, experiments in lactating rats have demonstrated that throughout lactation CRH gene transcript levels in the hypothalamus were significantly lower than that measured in control virgin cycling rats (Fischer *et al.* 1995). Thus, the decrease in circulating leptin at the onset of lactation may enhance appetite and food intake partly through the CRH pathway. Perhaps dairy cows that have a greater down-regulation of the CRH gene in response to decreased leptin concentrations are able to increase food intake more readily. Greater food intake would result in a greater supply of nutrients for milk production, thus may influence the 60 day lactation yield QTLs identified in the region of interest in the current study.

Neuropeptides B/W receptor 1 (NPBWR1)

NPBWR1 (neuropeptide B/W receptor 1) is a G protein-coupled receptor, which was originally identified by cloning of opioid-somatotropin-like receptor genes from human genomic DNA (O'Dowd *et al.* 1995, Schulz *et al.* 2007). Recently, two peptides named neuropeptide W (NPW) and neuropeptide B (NPB) were identified as the endogenous ligands for NPBWR1 (Shimomura *et al.* 2002, Tanaka *et al.* 2003). RT-PCR analysis of a number of human tissues showed a predominant central nervous system (CNS) distribution of NPBWR1 mRNA (Brezillon *et al.* 2003). *In situ* hybridisation showed high expression of NPBWR1 mRNA in various nuclei of the amygdaloid (located at the tail end of the caudate nucleus of the limbic system) and hypothalamus (Lee *et al.* 1999). Neuronal activation after ICV NPW injection was detected mainly in the hypothalamic nuclei, thalamus and brain stem (Levine *et al.* 2005). There was dense expression of NPBWR1 mRNA in the VMH, PVN and dorsomedial nuclei of the hypothalamus, all of which are strongly associated with modulation of feeding behaviour and metabolic disorders (Lee *et al.* 1999, Singh and Davenport 2006).

Physiological studies have shown that ICV or PVN administration of NPW into male rats caused a significant increase in short term food intake compared to controls (Baker *et al.* 2003, Levine *et al.* 2005, Shimomura *et al.* 2002). In contrast, NPW administration decreased feeding at the 24-hour time point and suppressed fasting induced food intake (Levine *et al.* 2005, Mondal *et al.* 2003). In mice, ICV administration of NPB caused an initial short term orexigenic effect followed by a more marked anorexic effect of NPB after 2 hours of ICV injection (Tanaka *et al.* 2003). Chronic ICV infusion of NPW for five days caused a persistent decrease in food intake and suppressed body weight gain, while body temperature, oxygen consumption and heat production were increased compared with saline-infused controls, suggesting NPW increases energy expenditure (Mondal *et al.* 2003). This is consistent with the findings that NPW suppresses food intake, ICV administration of anti-NPW IgG stimulated feeding (Mondal *et al.* 2003).

Rat NPBWR1 mRNA is expressed in the stomach and intestine (Tanaka *et al.* 2003). NPW-immunoreactive endocrine cells were abundant in the mucosal layer of the antrum in the rat, mouse and human stomachs (Mondal *et al.* 2006). Plasma NPW concentration of the gastric antrum decreased upon fasting and increased after re-feeding (Mondal *et al.* 2006) findings which as consistent with the hypothesis that NPW acts as a suppressant to feeding.

Male mice, with a targeted disruption of the gene encoding NPBWR1 (NPBWR1 $^{-/-}$), displayed a moderate, adult onset obesity, caused by increased food intake and decreased energy expenditure (Ishii *et al.* 2003). At 52 weeks of age NPBWR1 $^{-/-}$ mice had increased plasma concentrations of glucose, insulin and leptin compared with wild type mice. Leptin concentrations were disproportionately high for the degree of adiposity. Hypothalamic NPY RNA levels were decreased and POMC RNA levels were increased in NPBWR1 $^{-/-}$ compared with wild type mice, which is indicative of an underfed state (Ishii *et al.* 2003). Further investigation showed the mechanism of obesity was independent of leptin and melanocortin signalling (Ishii *et al.* 2003). The differences between NPBWR1 $^{-/-}$ and wild-type mice were only displayed in male mice.

ICV administration of NPW elevated plasma concentrations of prolactin and corticosterone and suppressed plasma growth hormone concentrations (Baker *et al.* 2003). Since these neuroendocine changes are also seen as acute responses to stress, these authors hypothesised NPW and NPBWR1 are a necessary signalling system for the expression of the hypothalamic response to stress (Baker *et al.* 2003). Results of a further study indicated that the receptors encoded by NPBWR1 are present on cells in the parvocellular PVN nucleus and perhaps on CRH-producing neurons in the nucleus (Taylor *et al.* 2005). Therefore, the central role of NPW to activate the hypothalamic-pituitary-adrenal axis is mediated by increased activity of CRH neurons (Taylor *et al.* 2005). Intraperitoneal injection of NPB into rats was found to increase plasma concentrations of thyroxine (T_4), parathyroid hormone (PTH), corticosterone and testosterone, while NPW increased plasma concentrations of ACTH, PTH, corticosterone, testosterone and oestradiol (Hochol *et al.* 2006).

As already discussed, food intake is an important factor in influencing milk yield. Cows that eat more produce more milk. The NPBWR1 gene encodes a receptor that mediates the actions of two peptides, NPW and NPB (O'Dowd *et al.* 1995, Schulz *et al.* 2007, Shimomura *et al.* 2002, Tanaka *et al.* 2003). A number of studies have investigated the effects of NPW and the current view is that this peptide decreases food intake and increases energy expenditure (Levine *et al.* 2005, Mondal *et al.* 2003, Tanaka *et al.* 2003). It is possible that the NPBWR1 gene is down-regulated during early lactation, which would reduce the suppressive affects of NPW on food intake enabling food intake to increase in response to the increase in nutrient demand caused by the onset of lactation. A major challenge to the hypothesis that NPBWR1 influences food intake in response to lactation is that the effects on energy homeostasis have only been demonstrated in males, females did not show significant effects of NPBWR1 knockout (Ishii *et al.* 2003. Experiments would need to be conducted in dairy cows to establish if NPBWR1 does have an effect in bovine females.

Summary

Four genes were discussed in this section due to their possible functions in some aspect of food intake control. Results of a study in which cows were fed a predominantly pasture diet showed that cows with similar metabolisable energy required for production had very different energy balances. The authors suggested that there was potential for selection of cows in early lactation to maximise intake to meet the energetic demands of production, therefore, minimise reliance on body reserves (Clark *et al.* 2005). A number of experiments have reported higher dry matter intake in high compared with low genetic merit cows (Kennedy *et al.* 2003, Kolver *et al.* 2005, Linnane *et al.* 2004, Roche *et al.* 2006b). The QTL in the region of interest are for milk, protein and lactose yields in the first 60 days of lactation and baseline IGF-I concentration. Opioid receptor, kappa 1 (OPRK1), proenkephalin (PENK), corticotropin releasing hormone (CRH) were considered the most suitable candidates due to their roles in control of food intake because the increase in nutrient requirements that accompanies the onset of lactation plays crucial role in greater milk production. The presence of the IGF-I QTL in this region also seems to fit with

this hypothesis due to plasma concentrations of IGF-I being an indicator of feed intake, energy balance and efficiency of feed utilisation (section 6.2). In addition, a positive relationship between plasma IGF-I concentration and energy balance has been reported in lactating dairy cows (Cisse *et al.* 1991, Ronge *et al.* 1988, Spicer *et al.* 1990).

6.7 Summary

A number of the genes discussed above have not previously been related to milk production in the literature. Each gene was reported to have multiple functions therefore they may affect milk production as well as other physiological processes. Recent research suggests that no one gene is responsible for a complex trait, such as the challenge response and milk production traits investigated in the study. Complex traits may be emergent properties of highly interconnected molecular networks that are modulated by many genes (Chen *et al.* 2008). The milk production and metabolic phenotypes identified in this study are likely to be the measurable outcomes of integrated metabolic processes controlled by hundreds or possibly thousands of genes. Thus, it is possible that that expression of a number of genes were up or down regulated in the region of interest to influence the QTL mapped to this region.

Some genes discussed in this study had a suggested function based on sequence similarity with a gene in another species. However, sequence similarity does not necessarily result in functional similarity. In addition, comparative map information was used to predict the genes in the region of interest. It is likely there would be differences between the list of genes generated in the current study using comparative mapping and a list of genes for the same region generated using the bovine gene map that is now being assembled. Furthermore, new information about genes and gene function becomes available daily in databases or in journal publications. Therefore, it is likely that new information will enable some of the candidate genes discussed in this study to be eliminated and identification of other candidate genes for the QTL in the region of interest.

The candidate genes discussed in this chapter have been considered for their relevance to milk production and metabolic QTL. Further work could investigate the relevance of these genes to other traits of importance such as reproductive performance. For example, the musculin gene may have a role in the poor reproductive performance of some high producing dairy cows. If musculin expression was up-regulated during early lactation decreasing protein synthesis in skeletal muscle, this could have a negative impact on blastocyst development as a mouse model showed over expression of musculin is lethal to early mouse embryos (Yu *et al.* 2003).

Due to the very detailed investigation conducted into the region of interest on BTA14 it was not feasible to investigate more than one region on one chromosome. Many chromosomal regions that were strongly associated with certain QTL were identified (Chapter 5). These interesting chromosomal regions should be investigated in future research.

CHAPTER 7

General Discussion

The focus of the research described in this thesis was to investigate ways to improve the capture of the genetic potential for milk production and increase the rate of genetic gain in dairy cattle above that which is achieved using current methods of animal evaluation (Chapter 2).

The onset of copious milk synthesis and secretion is accompanied by changes in physiology that partition nutrients towards milk production. Numerous studies have examined concentrations of metabolites and hormones under a number of experimentally induced challenge conditions in dairy cattle. The objective was to determine physiological markers that could be measured in calves of both sexes to identify animals of superior genetic merit for milk production traits (Woolliams and Løvendahl 1991). However, very few studies have reported the selection of animals based on physiological traits (Chapter 2). Thus, attempts to identify physiological indicator traits that could be used to select high genetic merit animals of both sexes, for milk production traits at an early age were not successful. Despite this, hormones and the physiological pathways they influence are vital for determining variability between animals in their ability to produce milk. Determining genetic markers for these physiological traits could avoid the influence of environmental effects and therefore provide a more accurate means of indirectly selecting animals with superior genetic merit for milk production.

The current study combined previously used challenge methodology with the more recent QTL mapping technology. The intention was discover QTL that are associated with genes that underlie some of the important metabolic pathways involved in milk production. The overall aim of this thesis was to explore the use of metabolic phenotypes as a means of improving the rate of genetic gain in dairy cattle.

7.1 The challenges

The challenges were chosen in an attempt to emulate aspects of metabolism that have important roles in supplying nutrients for lactation. The individual challenges selected were deemed the most promising based on current understanding of the biology of the dairy cow and the experience of others reported in the literature. The adrenaline challenge was used because the

responses were expected to provide information on the animals ability to mobilise triacylglycerol from adipose tissue and glycogen from the liver. Mobilisation of lipid reserves may affect the ability to sustain high levels of milk production in dairy cows, particularly during early lactation when intake does not increase sufficiently to meet nutrient demands of lactation. The glucose challenge was used because the response was expected to reflect that animals ability to control blood glucose concentrations and metabolise carbohydrates. Carbohydrate metabolism has a critical role in providing glucose for lactose synthesis in the lactating cow. The TRH challenge was used because the responses were expected to reflect the control of hormones important to energy homeostasis and mammary gland development. Responses to metabolic challenges are confounded with energy balance if they are conducted during lactation. Thus, challenges were conducted when the heifers were approximately 18-months old and in early pregnancy to minimise the complications caused by lactation in interpreting physiological data (Chapter 2).

There was significant between-sire variation in all the response variables measured, except for NEFA response to the TRH challenge. This suggests that there is a genetic component to the control of the metabolic traits measured and provided confidence for continuing on to conduct QTL mapping (Chapter 4).

When conducting an experiment that involves the collection and processing of a large number of samples from a large number of animals it is inevitable that a very large data set will be generated. It is essential that the logistics of collecting these data a carefully planned and processes are put in place to assess and maintain the integrity of the data. In addition, it is essential that statistical checks are carried out to ensure systemic errors are identified and if possible corrected prior to commencement of data analyses (Chapter 3).

The decision to keep heifers outdoors on pasture during the challenge closely simulated standard management practices for dairy heifers in New Zealand. However, this did introduce variability between experimental groups in quantity and quality of feed as well as variability in

environmental conditions (Chapter 4). The effect of environmental factors on response variables could have been better accounted for in the statistical analyses if data on the quantity and quality of feed preceding the challenges and a complete set of local climatic conditions during the challenges had been measured. These data should be collected in future experiments.

Selection of appropriate response variables is vital for the detection of genetic differences and therefore QTLs (Chapter 4 and Appendix E). The metabolic phenotypes calculated from the glucose challenge data gave an indication of sensitivity of the pancreas to glucose, the sensitivity of the adipose tissues to insulin, and glucose clearance. The metabolic phenotypes calculated from adrenaline challenge data quantified the rate and quantity of NEFA and glycerol released from adipose tissues and glycogen released from the liver. The metabolic phenotypes generated from the TRH challenge response variables provided an indication of the ability of the heifers to release GH, prolactin and TSH into the circulation.

Although there were some minor problems with the data, these data are the definitive data set for 18-month-old, early gestation heifers. No other data set of this size and on this age of animal has been reported. Thus, these data are an invaluable resource for meeting two objectives. The first was for generating metabolic phenotypes that can be used for QTL searches for production traits. The second was to obtain a deeper insight into the physiology and its genetic variability in a group of animals in a similar physiological state, heifers in early gestation, and ascertain whether these metabolic phenotypes were related to subsequent lactation performance. Thus, while the pattern of the responses to the various challenges are consistent with those observed in previous experiments (Kolver *et al.* 2001, Løvendahl and Sejrsen 1993, Mackenzie *et al.* 1988, Xing *et al.* 1991) and with knowledge of basic physiology, the potential to correlate the metabolic phenotypes to the genotype of such a large number of animals may be extremely valuable.

7.2 Mapping QTL

An initial whole genome scan was conducted by genotyping 1679 animals for 283 microsatellite markers, obtained primarily from published marker maps (Chapter 5). 581 QTL were significant at the 1% level and 275 of them were QTL of metabolic phenotypes (Appendix F). From the large number of metabolic QTL it is clear that the challenges conducted did perturb the physiology of the animals in such a way that it allowed control of metabolic processes to be indicated at a genetic level. However, QTL for baseline measurements were also identified, thus, in these cases the system did not need to be perturbed.

There were a large number of clusters of similar QTL, either metabolic or milk production QTL, that were co-located. Certain phenotypes were strongly associated with particular chromosomes, for example clusters of QTL for insulin response phenotypes were found on chromosomes 9, 15, 18 and 25. These regions deserve further investigation as they may harbour important regulatory genes for energy metabolism in the dairy cow. They may also have implications for human health research, in particular relating to the insulin QTL and their possible role in diabetes.

An objective of this study was to identify chromosomal regions where metabolic and milk production QTL were co-located. It was anticipated that these regions would contain genes that had a significant impact on the control of milk production. However, a major finding of this study was that there were very few chromosomal regions in which both metabolic and milk production QTL were co-located (Chapter 5). Thus, neither pleiotropy nor genetic linkage appeared to occur between metabolic and milk phenotypes for the majority of chromosomal regions identified by the QTL mapping conducted in this study. Perhaps this is not a surprising finding, given the difficulty in establishing consistent and repeatable physiological indicators of milk production demonstrated in previous studies (Woolliams and Løvendahl 1991).

The findings of the current study suggest that hormone and metabolite concentrations in heifers during growth and gestation are not indicative of subsequent milk production at a phenotypic

level. Thus, while some phenotypic correlations calculated using data from the current study were significant (*P* < 0.05), they were all low (R < 0.15) (Appendix I). Furthermore, previous reports have shown few phenotypic associations between milk yield and pre-partum or pre-pubertal plasma hormone and metabolite concentrations (Lacasse *et al.* 1994, Mäntysaari *et al.* 1999, Taylor *et al.* 2006). The lack of a relationship between the metabolic and milk production phenotypes may be due to differences in physiological state of the animals when the phenotypes were measured. The metabolic phenotypes were measured in 18-month-old, early-gestation heifers that were growing as well as pregnant (anabolic), while the milk production phenotypes were measured in lactating, mature animals (catabolic). Further, it has been suggested that maiden heifers and lactating cows may differ in the quantitative and qualitative expression of their genes because of the differences in their physiological state (Veerkamp *et al.* 2003). Genetic correlations between metabolic and milk production phenotypes were not calculated in the current study. However, these data would be suitable for calculating genetic correlations and further research should include such calculations.

The control of gene expression is highly complex and some aspect of control may be remote from the gene of interest. Therefore, it is possible that when clusters of metabolic and milk production QTL occur in distinct regions on the same chromosome, the genes underlying the metabolic QTL may influence the genes underlying the milk production QTL by to long-range regulation of gene expression (Chapter 5). Co-regulation of genes on different chromosomes also needs to be investigated, for example, milk protein genes on different chromosomes may share common control mechanisms (Ollivier-Bousquet 2002). Thus, long-range regulation of gene expression will need to be considered in future QTL studies (Kleinjan and van Heyningen 2005). Other types of analyses will be needed to investigate the complex interactions and networks that control gene expression and function.

7.3 Usefulness of metabolic challenges

It may be that the choice of challenges was not appropriate to locate chromosomal regions that contain QTL for most of the milk production traits. Alternatively, it may be that the concept

that it is possible to detect milk production QTL by identifying metabolic phenotypes is wrong. Confidence in the techniques used to identify QTL and their relationships with phenotypes in the current study is warranted given that similar regions have been identified in other studies. For example, in the current study milk fat percentage QTL were mapped to 0cM on BTA14. This chromosomal region, milk production QTL and the gene most likely responsible, DGAT1, have been reported previously (Grisart *et al.* 2002). The DGAT1 gene encodes the acyl CoA:diacylglycerol acyltransferase 1. This protein has a number of functions related to lipid physiology and in particular catalyses the attachment of the third fatty acid to complete triacylglycerol formation (Cases *et al.* 1998). Adrenaline causes lipolysis, consequently an adrenaline challenge would not give an indication of triacylglycerol formation. The challenges applied in this study were directed towards the control of metabolic process, which is also true for most challenges reported in the literature (Chapter 2). Measurement of hormones and metabolites in plasma samples are unlikely to provide information of specific steps in biochemical pathways. Therefore, the challenges chosen were not able to identify many of the QTL for milk production. Nevertheless, the metabolic phenotypes and QTL identified may still be useful, particularly for improving understanding of the control of metabolic processes.

At the commencement of this study a diagram was presented (Chapter 2) that attempted to integrate physiological changes that support lactation. However, control of quantitative traits, such as milk production, is by complex networks that are likely to be influenced by hundreds or possibly thousands of genes (Figure 7.1 and Figure 7.2). Thus, understanding physiological systems can not be achieved by consideration of a subset of metabolic pathways. Rather they must be viewed as a complex set of integrated networks that control metabolism. Metabolic networks and the rapidly increasing genetic and biological information are too much for the human brain to comprehend. As a result the discipline of computational biology has evolved and numerous websites, such as KEGG (Kyoto Encyclopedia of Genes and Genomes) and Ingenuity Pathways Analysis, have been developed. These websites mine genetic and biological information in the literature and integrate it into a useable network form.

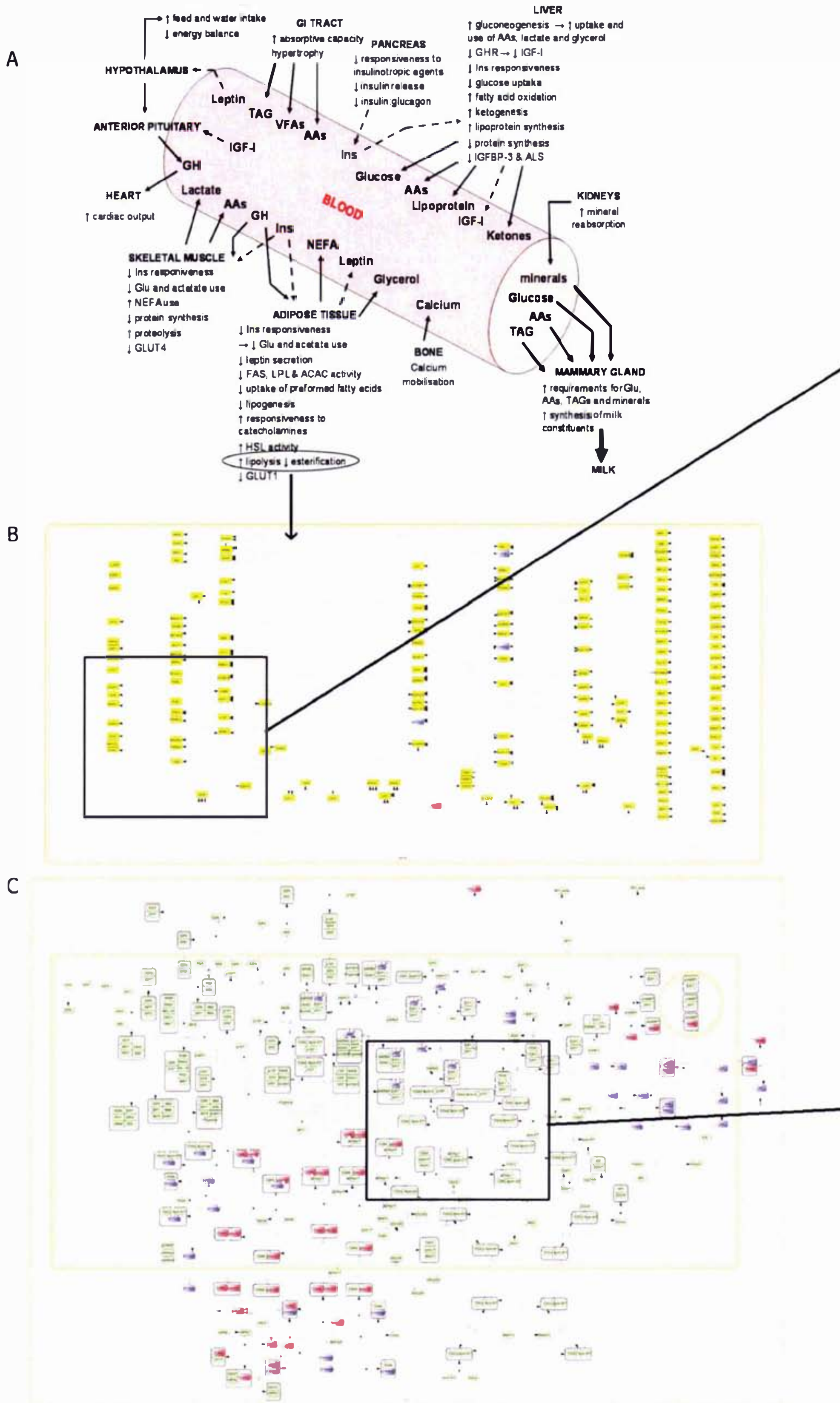
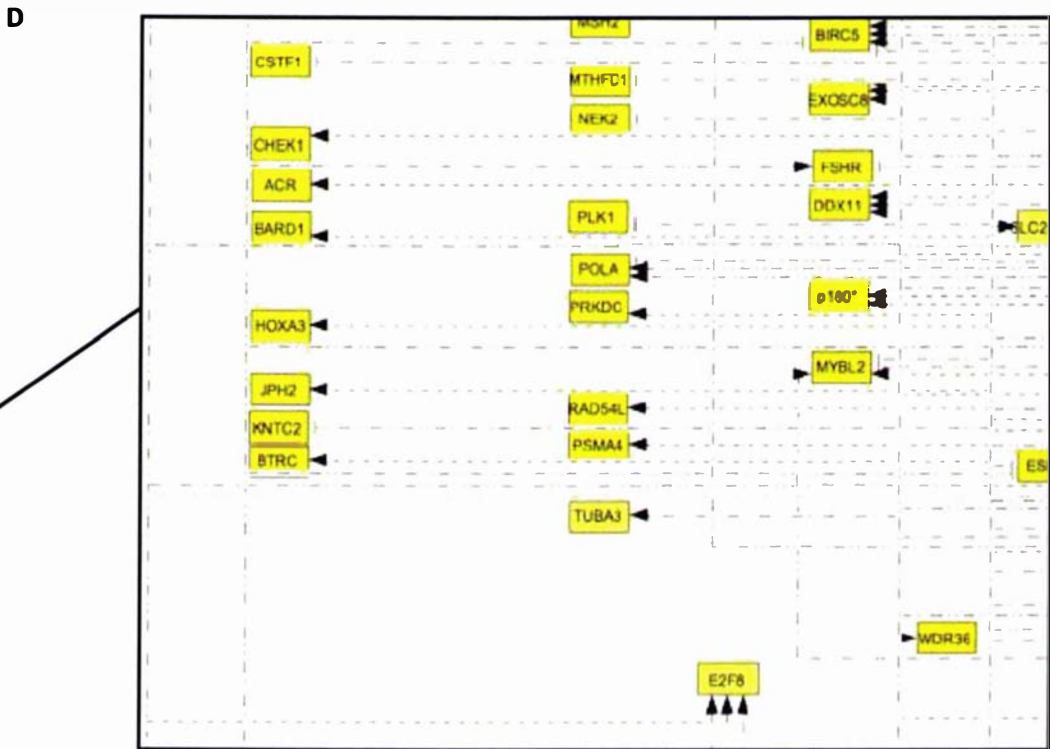


Figure 7.1 (A) The diagram constructed in the literature review of this thesis. Illustration of the type of networks involved in just one part of the original diagram (B) a map of target gene network and (C) a map of protein to protein interaction network. B and C are taken from Calzon *et al.* (2008).

D



E

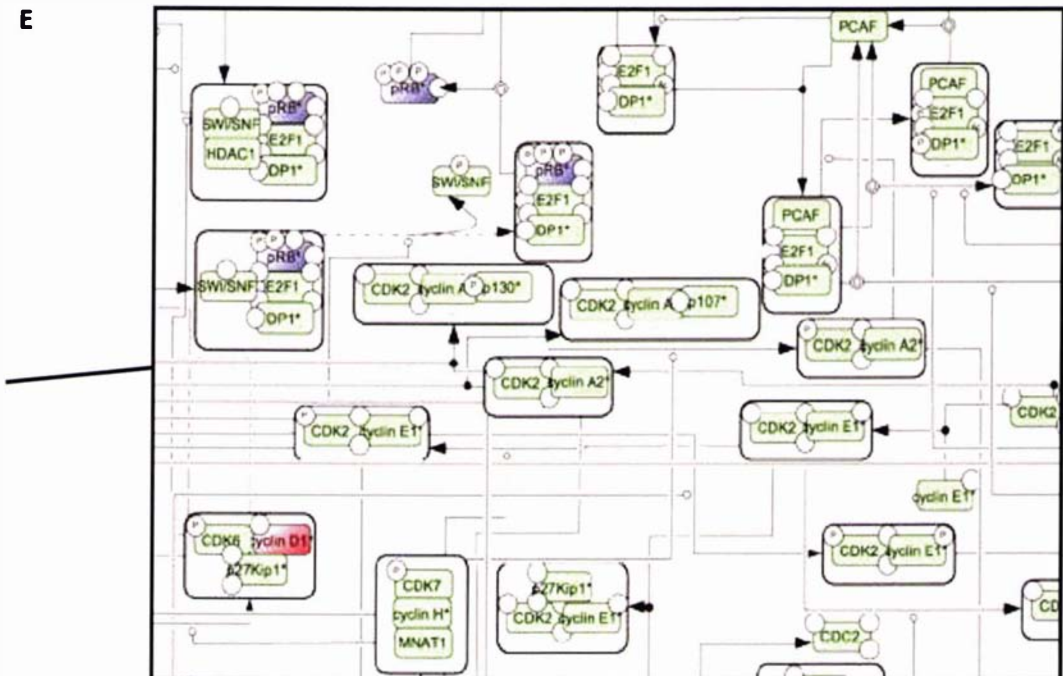


Figure 7.2 Enlarged images of the corresponding boxes on the network diagram presented in Figure 7.1. (D) Map of target genes of E2F transcription factors. Each E2F associates with different cofactors to activate or inhibit the transcription of many genes (yellow boxes): pointed arrows mean activation, flat arrows mean inhibition. (E) Map or protein to protein interaction network. Tumour suppressor proteins in blue, oncogenes in red and other proteins in green. Several proteins inside a box is a complex. Solid circle to an arrow is an association, double circle to an arrow is a disassociation, a line from a protein or complex that ends in a circle is catalysis Taken from Calzon *et al.* (2008).

It is still feasible that the control of metabolic processes, which are themselves likely to be controlled by numerous genes, are important factors influencing milk production. However, they will only comprise one part of a complex molecular network modulated by the expression of many genes, therefore co-location of milk production and metabolic QTL would seem unlikely. The original diagram of the control of lactation needs to be expanded to take into account the complex nature of interactions that occur within a metabolic network and the genes that influence this network.

There are a vast number of proteins and control points associated with gene expression and functions of gene products. These include transcription factors that either activate or repress transcription, transcription co-regulators, RNA polymerase complexes, the splice variants produced, microRNA, translation initiation factors, post-translational modifications and protein to protein interactions (Calzon *et al.* 2008, Rosenfeld *et al.* 2006, Van Tassell *et al.* 2007, Vary and Lynch 2007). It is through the use of molecular networks that all aspects of control can be integrated to develop a more accurate understanding of the system (Calzon *et al.* 2008, Chen *et al.* 2008).

Perhaps there is still a use for metabolic challenges in the future if a more sophisticated assessment of responses to the challenge could be used. Rather than measuring changes in concentrations of metabolites and hormones in blood, gene expression could be measured in various tissues such as the liver and adipose tissue. Gene expression studies could then be conducted on the same tissues from lactating animals allowing the construction of co-expression networks with the intention of finding causal relationships with milk production traits.

7.4 QTL and genomic selection

Marker assisted selection has been applied in some dairy cattle breeding schemes but to date has only resulted in minor improvements in genetic gain (Spelman *et al.* 2007). Difficulty in developing reliable and effective tests from QTL results was due to inadequate approaches to finding quantitative trait nucleotides. These include fine mapping with limited markers,

comparative mapping, and candidate gene/marker associations (Van Tassell *et al.* 2007). If marker information from a sparse marker map were to be included as selection criteria in an outbreeding species, the linkage phase between a marker and a QTL would have to be established for every family in which the markers were to be used. Consequently, this type of marker information is of limited use (Meuwissen *et al.* 2001). As a result of the above, the promise of molecular information in ruminant genetic improvement schemes has not yet been achieved.

Genetic marker technology has advanced dramatically since the commencement of this study. The use of dense marker maps for genomic selection was first proposed in 2001. It was suggested that predicting breeding values using genomic information with a marker spacing of 1 cM would enable an accuracy of selection of between 0.73-0.85 (Meuwissen *et al.* 2001). Since that time, sequencing of the bovine genome has been completed, which has resulted in a large number of single nucleotide polymorphisms (SNP) being placed in the public domain. This combined with the considerably lower cost of conducting SNP genotyping compared with genotyping using microsatellite markers will result in a dramatic increase in the utilisation of genetic markers for breeding schemes (Spelman *et al.* 2007).

A 7,500 SNP chip is now available for the Holstein-Friesian by Jersey crossbred cows used in the current study and a genomic selection experiment with 3000 bulls using a 60,000 SNP chip is currently being conducted (Spelman *et al.* 2007). These maps will enable vastly greater power in identifying QTL than the 283 microsatellite marker map used in the current study. Therefore, the metabolic phenotypes derived in this study need to be re-run against the new SNP chip data to obtain more accurate QTL locations.

As the density of the SNP maps continue to increase the power of the studies will also continue to increase and identification of markers for desirable phenotypes will be possible with very small numbers of animals. Technically it will be possible to have a complete genome sequence of every individual animal. The limitation will then become the ability of the scientists dealing

with these vast amounts of data to envisage ways in which to effectively use the information. It is anticipated that in the New Zealand situation, SNP chips will be used to screen animals. The most likely cohort to be screened will be bull calves born from artificial insemination. Providing the accuracy of selection using the SNP is moderate, the rate of genetic gain may be increased by 75-80% due to increased selection intensity and decreased generation interval (Spelman *et al.* 2007). In time, formal progeny testing of young bulls may disappear due to the expense associated with this process. However, bulls will be retrospectively progeny tested when their daughters are milked in commercial herds, providing an accurate animal identification and herd testing system remains in place.

An exciting new era in animal genetic improvement is about to commence due to the application of SNP markers. The use of SNP associations does not require knowledge of the genes being selected for. This raises the question of whether there is a need to know the genes and their functions that underlie traits of interest. Current methods of animal evaluation and selection have resulted in rates of genetic gain of about 1% of the trait mean per year. Such slow rates of change are likely to allow the population to balance genetic gains in the traits of economic importance with natural selection on the traits that affect the general wellbeing of the animals. If more intense selection is placed on economic traits through the use of SNP chips, it may be that balancing selection will not have the opportunity to work on important (but undefined) adaptive traits. For example, if some of the genes identified in Chapter 6 are driven to homozygosity by intense selection, the question at least ought to be asked as to whether it is desirable for the long-term health of the population. Maybe some fundamentally important cellular functions will be upset. From a scientific perspective, research will most likely continue into searching for the genes that underlie the QTL identified via SNP chips. This is likely to involve the construction of molecular networks and gene atlases, which include all transcript types expressed in the total RNA of a cell, to elucidate the control of complex traits. However, such research may take a decade, whereas the application of SNP-based selection is almost certain to begin in the New Zealand dairy industry in 2008. There is a danger that the

rapid commercial exploitation of this new science may inadvertently lead to genetic ill-health in the national dairy herd.

7.5 Conclusion

The overall aim of this work was to explore the use of metabolic phenotypes as a means of improving the rate of genetic gain in dairy cattle. The research has shown that metabolic challenges did not provide useful information for the detection of QTL for milk production traits. This is likely to be due to the highly complex and integrated molecular networks controlled by many genes that influence complex milk production traits. The data generated in this programme of work will be suitable for more advanced examinations of the genetic control of milk production using the new generations of SNP chips.

CHAPTER 8

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CHAPTER 9

Appendices

Appendix A: Catheterisation Procedures

Consumables

Plugs

Injection site cap (Zuellig #423801) – these will be attached to the catheter extensions during the periods from insertion to the start of the glucose challenge, and overnight after the end of the glucose/adrenaline challenge day. They have a rubber diaphragm for injection which allows a solid column of heparinised saline to be placed in the catheter between sampling periods, and reduces the risk of development of blood clots, fibrin tags, and embolism should these detach.

Catheter extension (lazy line)

Catheter extension with slider clamp (Zuellig #403380) with injection site cap (Zuellig #423801) – a commercial plug and 22cm lazy line system which can be attached to the catheter and acts as an extension/lazy line for use during the intensive sampling periods. The injection site cap allows samples to be taken without removing it. This equipment reduces the risk of air embolism and contamination of the catheter while facilitating access when working in close contact with the animals.

Saline

Standard saline solution (0.9% NaCl #1324 sterile 1L: Zuellig) from flexipacks will be used for flushing catheters after infusion of the various infusates and also after infusion with Bivatop 200.

Heparinised Saline

The lazy line and catheter assemblies will be flushed with heparinised saline. Fresh heparinised saline was prepared at the beginning of each challenge day. Heparin (25,000IU/ml, Zuellig) will be added to flexipacks of saline to give a final concentration of 50IU/ml saline. The date the heparin was added and the actual concentration was written on each flexipack. A vaccination gun with no needle was attached to each bag of heparinised saline to enable accurate and efficient dispensing of heparinised saline into the catheter assembly after each blood sample was taken.

Other requirements

Equipment:

Clippers (including brushes for cleaning, clipper oil etc.)

Tackle boxes to hold all the equipment required for catheter insertion

Tackle box to hold "emergency kit" including: pliers; scissors; rat tooth forceps; xylazine 2%, with syringes and needles for its injection; notebook for recording use of xylzine and reinsertion of catheters.

Drugs:

Biocil surgical scrub (iodine based antiseptic) scrub brushes

10% povidone iodine in alcohol

Lignocaine (2%)

Xylazine (2%)

Bivatop 200

Betadine ointment

Consumables:

Glue (Loktite Gel)	Catheters
Elastoplast 7.5cm	Injection site caps
Elastoplast 10cm	Lazy lines
Syringes 3ml	Needles 16g x 1"
Syringes 10ml	Needles 18g x 1"
Syringes 20ml	Needles 20g x 1"
Vaccinator guns for dispensing saline	

Insertion procedure

Teams

Teams of 3 people performed catheter insertions: an animal restraint person (held the heifers head while the catheter was being inserted); a catheter insertion technician; an assistant to prepare injections, bandages etc and hand these to the insertion technician as required.

Restraint

A halter was put on the heifer in the yards, she was then directed into a crush where the animal was restrained in the head bail. A backing bar was pushed up behind the animal and at the same time the head was pulled forward and the halter tied to a bar in front of the crush so that the head and neck were in an outstretched position. Lateral pressure was applied with the crush. At this time the animal should be restrained in such a way that they only had limited head movement up and down and were held firmly by the crush. Some animals struggled at this stage and small number required sedation, in some cases if the lateral pressure of the crush was released slightly this was sufficient to calm the animal. The catheter team's restraint person then held the animals head.

Sedation

Animals were not sedated as a routine part of the catheter insertion procedure. The need for sedation was based upon the animal's behaviour once it entered the crush. If the animal exhibited a strong anxiety response (excessive thrashing of the head and or body) to the catheter insertion procedure then the animal was sedated. Sedation was with 2% xylazine administered intravenously through the tail-vein at a dose rate of 0.1ml per 100 kg live weight.

Preparation of surgical site

An area (approximately 10 x 15 cm) on the neck was clipped free of hair. A circular area that extended approximately 10 cm from the site of catheter insertion was scrubbed with povidone iodine soap, washed with water then a solution of 10% povidone iodine in alcohol was applied. The cleaned site was left for one minute before the catheter was inserted. If the site was contaminated, for example, by the animal lying down, then it was cleaned again.

Injection of local anaesthetic

The intended site of injection was identified by raising the jugular vein, by applying pressure to the vein, in the most ventral part of the prepared surgical site. Approximately 6ml of local anaesthetic was injected subcutaneously into the intended site so that a bleb developed just dorsal of the jugular vein.

Bandage preparation

Pieces of Elastoplast bandage were prepared, one 15 cm piece of 10 cm wide bandage with a hole at 1/3 of the way along the 15 cm in the middle width ways (doughnut), and a 5 cm piece of 7.5 cm wide bandage with a 3 cm deep "v" notch cut into each side for a butterfly attachment. A diagram of these bandages is shown in Figure 9.1.

Syringe preparation

Prepared three syringes; one with the appropriate amount of Bivatop 200 for the weight of each animal; one with 20ml of heparinised saline; and one empty syringe to use for checking the catheter is working.

Lazy line preparation

The catheter extension and catheter plug were removed from their packaging, and the catheter plug was attached to the catheter extension. The extension assembly was filled with heparinised saline.

Catheter insertion

The person inserting the catheter thoroughly scrubbed their hands and arms with povidone iodine scrub soap. The jugular vein was raised by pressing their thumb on the vein, below where the anaesthetic bleb was situated, and then inserted the catheter by pushing the catheter down through the skin and bleb of anaesthetic into the vein. Once the vein had been entered, the needle assembly was loosened and the needle drawn back slightly to remove the sharp point, then the cannula was slid into the vein to its full length. The needle was removed and the extension and catheter plug were attached to the catheter. Blood was pulled into the catheter extension to confirm that the catheter was working and then 5 ml of saline was infused.

Securing the catheter

A small bleb of iodine cream was applied over the insertion site, then the butterfly body was slid behind the plastic top of the catheter and a small amount of glue was applied between the hub and the butterfly body. The butterfly bandage was attached by twisting the butterfly body (Figure 9.1) around the plastic top of the catheter and then gluing the wings of the butterfly bandage to the skin of the animal. Then the plug and catheter extension were threaded through the hole in the doughnut and the doughnut was glued over the butterfly and to the skin of the animal. At this point it was confirmed that the catheter was in the vein by drawing blood into the extension line. Then the prepared dose of Bivatop was administered followed by 5 ml infusion of heparinised saline. The heifers' neck was wrapped in Elastoplast with the catheter extension sitting between two layers of bandage and the insertion site covered. The heifer was then released from the crush.

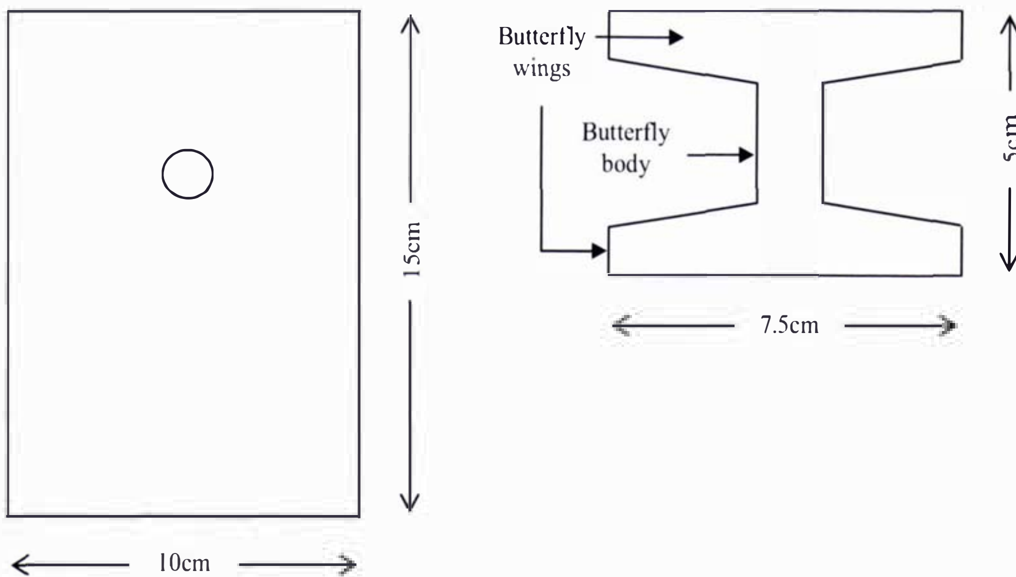

Figure 9.1 Diagrams of the bandages used to secure the catheter to the animal. Not drawn to scale. The diagram on the right is the 'doughnut' and on the left is the 'butterfly' bandage.

Appendix B: Infusate preparation

Adrenaline

Weight of Adrenaline calculations

Required concentration: 80μg/ml   Dose rate: 1.4μg/Kg live weight (LW)

Required Volume of Solution: 300ml

Correction Factor based on molecular weight (MW) difference:

= MW of Adrenaline HCL / MW of Adrenaline pure

= 219.7 / 183.2

= 1.199 (Correction factor)

Actual Concentration = Required Concentration x Correction Factor

= 80μg x 1.199

= 95.92μg

Actual Concentration (Conversion of μg to g) x 300ml

= (95.92μg/1000000) x 300ml

= .029g per 300ml

Thus use 0.029g of Adrenaline HCl per 300ml of saline.

Live Weight calculation

$$\frac{\text{Dose Rate x Live Weight of Animal (LW)}}{\text{Required Concentration}}$$

For example:

LW is 350Kg

Dose Rate is 1.4μg/Kg LW

Required Concentration is 80μg/ml

$$\frac{\text{Dose Rate x Live Weight of Animal (LW)}}{\text{Required Concentration}} = \frac{1.4 \times 350}{80}$$

$$= 6.125\text{ml}$$

Adrenaline preparation

Adrenaline HCl is soluble in water at 80μg/ml but is unstable at neutral or basic pH. Adrenaline HCl solution is a clear colourless to light yellow solution and darkens in colour as it degrades. Adrenaline HCl solution is light and air sensitive, thus was made up the night before or the day of use to avoid any degradation.

1. Pre-measure out 300ml of saline solution into a glass-measuring cylinder.
2. Using a glass weigh boat, weigh 0.029g ± 0.0001g (4dp) Adrenaline HCl. Record the actual weight taken on the weighing sheet
3. Transfer the weighed adrenaline HCl to a 500ml Pyrex beaker rinsing with the pre-measured saline solution. If any of the powder remains on the weigh boat, flush with saline using a 1ml plastic disposable pipette.
4. Add 0.025g of L-Ascorbic Acid (acts as an antioxidant) to the Adrenaline HCl solution.
5. Moderately stir the adrenaline solution by swirling for two minutes to ensure total solubility, as adrenaline dissolves readily in the saline.
6. Using the live weight of the individual animal calculate the amount of solution required using the LW calculation below. Withdraw from the beaker the required solution into a 10ml syringe (Approximately 6.1ml based on a live weight of 350kg). Place syringe guard on the end of the syringe.
7. Wrap the syringe in tinfoil and apply sticky label. The labels will have the animal number, mob, group, the volume of the infusate, and name of the challenge. (The solution is light and air sensitive and will darken as it degrades.)
8. Store prepared syringes in a chilly bin in an up right position and kept at 4°C.
9. Repeat steps 1 to 8 for all animals involved in the trial, as well as preparing three extra syringes as a contingency plan, this will contain the maximum volume of infusate, used in that group and the volume will be recorded on the syringe.

10. Withdraw 4mls of Adrenaline HCl solution into a 5ml vial. Wrap in tinfoil. Label as Adrenaline HCl museum sample 1, batch number and date. Place into the –18°C freezer for QA analysis at a later date. Repeat the process for museum sample 2.
11. Withdraw 4mls of Adrenaline HCl solution into a 5ml vial. Wrap in tinfoil. Label as Adrenaline HCl museum sample 3, batch number and date. Place in the chillibin with the other syringes. Return vial from field and place into the –18°C freezer for quality assurance (QA) analysis at a later date. Repeat the process for museum sample 4.

Glucose

Glucose was supplied in 500ml flexibags at the required concentration. To get the required volume of glucose for each animal the excess solution was removed from the 500ml flexibags.

Glucose calculations

Required concentration: 40% (already supplied in flexibags)

Dose rate: 0.3g/kg live weight   Density of glucose: 1.150

Weight of empty flexibag: 45g   Volume of glucose in flexibag: 500ml

Volume of glucose left in tube: 10.3ml

Weight of glucose left in tube: 11.85g

Volume of glucose required: (Dose rate x Live weight)/concentration

Volume of glucose to be removed from flexibag:

Volume of glucose in bag – volume of glucose required

Weight of glucose to be removed:

(Volume of glucose to be removed x density of glucose) – weight of glucose left in tube

Weight of bag after glucose removed:

(volume of bag x density) – weight of glucose to be removed + weight of empty bag + weight of glucose left in tube

Example: Volume of glucose required

(Dose rate x live weight)/Concentration

(0.3 x 400kg)/0.4 = 300ml

Volume of glucose to be removed: (500ml – volume of glucose required)

500ml – 300ml = 200ml

Weight of glucose to be removed:

(Volume of glucose to be removed x density of glucose) – weight of glucose left in tube

(200ml x 1.15) – 11.85g = 218.15g

Weight of bag after glucose removed:

(Volume of bag x density) – weight of glucose to be removed + weight of empty bag + glucose left in tube

(500 x 1.15) – 230 + 45 + 11.85 = 401.85

Glucose preparation

1. The weight of glucose to be removed from each bag was calculated using the above formulae.
2. An empty beaker was placed on a balance and the balance tared
3. The plug of the glucose flexibag was undone and glucose solution was poured into the beaker.
4. The glucose flexibag was reweighed to check the correct amount of solution was in the flexibag. The weight of the glucose flexibag was recorded on a glucose weigh sheet.
5. The glucose solution removed from the flexibag was discarded.
6. A label with the animal number, mob, group, volume of infusate and name of challenge was stuck on the flexibag.
7. The flexibag was placed in a chillibin and stored at 4°C overnight
8. The above steps were repeated for all animals involved in the trial. Two additional 500ml flexibags, a 50ml syringe and a sterile needle were supplied for each mob so that glucose could be withdrawn in the field.

9. For each mob, two museum samples were collected. From one flexibag 4ml of glucose solution was removed and put into a 5 ml vial and placed into a -18°C freezer for QA analysis at a later date, this process was repeated for museum sample 2. These museum samples were labelled with the challenge group, museum sample number, batch number and date.

10. From the same flexibag, two more museum samples were obtained. These were also 4 ml samples placed in 5 ml vials. These vials were placed in the chillibin with the flexibags, once they were returned from the field they were places in a -18 °C freezer for QA analysis at a later date. These museum samples were labelled with the challenge group, museum sample number, batch number and date.

TRH

Weight of TRH calculations

Required concentration: 15μg/ml

Dose rate: 0.3μg/Kg live weight (LW)

Required Volume of Solution: 300ml

(Required Concentration x 300)/Conversion of μg to g

= (15μg x 300)/1000000

= 0.0045g/300ml

Thus, use 0.0045g of TRH per 300ml of saline.

Live Weight calculation

$$\frac{\text{Dose Rate} \times \text{Live Weight of Animal (LW)}}{\text{Required Concentration}}$$

For example: LW is 350Kg

Dose Rate is 0.3μg/Kg LW

Required Concentration is 15μg/ml

$$\frac{0.3 \times 350}{15} = 7\text{ml}$$

TRH preparation

TRH is soluble in water or aqueous buffer at 10mg/ml. TRH solution is clear or colourless and can be stored for up to 30 days refrigerated without loss of activity or potency. Although there is no indication of light sensitivity, it would be prudent to protect solution from the light.

1. Pre-measure out 300ml of saline solution into a glass-measuring cylinder.
2. Using a glass weigh boat, weigh 0.0045g ± 0.0001g (4dp) TRH. Record the actual weight taken. Transfer the weighed TRH to a 500ml Pyrex beaker rinsing with the pre-measured saline solution. If any of the crystals remains on the weigh boat, flush with saline using a 1ml plastic disposable pipette.
3. Moderately stir the TRH solution by swirling for two minutes to ensure total solubility, as TRH dissolves readily in the saline.
4. Using the live weight of the individual animal calculate the amount of solution required using the LW calculation below. Withdraw from the beaker the required solution into a 10ml syringe (approximately 7ml based on a live weight of 350kg). Place syringe guard on the end of the syringe.
5. Wrap the syringe in tinfoil and apply sticky label. The sticky labels will have the details of the live weight, animal number, the volume of the infusate in syringe, type of solution and name of the challenge.
6. Store prepared syringes in a chilly bin in an up right position and kept at 4°C.
7. Repeat steps 1 to 6 for all animals involved in the trial, as well as preparing three extra syringes as a contingency plan, this will contain the maximum volume of infusate, used in that group and the volume will be recorded on the syringe.
8. Withdraw 4mls of TRH solution into a 5ml vial. Wrap in tinfoil. Label as TRH museum sample 1, batch number and date. Place into the –18°C freezer for QA analysis at a later date. Repeat the process for museum sample 2.

9. Withdraw 4mls of TRH solution into a 5ml vial. Wrap in tinfoil. Label as TRH museum sample 3, batch number and date. Place in the chillibin with the other syringes. Return vial from field and place into the −18°C freezer for QA analysis at a later date. Repeat the process for museum sample 4.

Appendix C: Iodination procedures

Iodination of bovine GH using Iodogen

IODOGEN is an oxidising agent capable of converting iodide to a more reactive form. The procedure is simple requiring only mixing of solutions of the protein, sodium iodide(I^{125}) and iodogen. The reaction is terminated by the addition protein buffer.

Iodination components

PO_4 Stock Solution (0.5M PO_4)

7.80g $NaH_2PO_4 .2H_2O$ in 100 mls distilled water. Name 'A'.

35.51g Na_2HPO_4 anhydrous in 500 ml distilled water. Name 'B'.

(or 44.5g $Na_2HPO_4 . 2H_2O$)

Add 82mls of 'A' to the 500mls of 'B' , adjust to pH 7.4

Store as 40ml aliquots in screw top jars at -20°C.

This becomes the PO_4 stock solution.

0.05 M PO_4

Take PO_4 stock solution and dilute 1:10 in DI water.

Make up 100mls for iodination.

Elution Buffer (200mls)

20mls PO_4 stock solution

0.4g BSA

0.2g NaN_3

DI water

Adjust pH to 7.4

Make up to 200mls

Iodogen

Iodogen (1, 3, 4, 6-tetrachloro-3α, 6α diphenyl glycoluril; Pierce and Warriner, NZ Ltd; 100μl of a 100μmol/l solution in dichloromethane) was added to a polypropylene vial and the solvent evaporated.

Precipitation Buffer

2mls of Stock PO_4

1.861g EDTA (disodium,dihydrate)

1g BSA

0.1g Sodium Azide

Make up to 100mls with Milli Q water

TCA

10% Tricloroacetic acid

Make up to 200mls in deionised water.

Label (I^{125})

0.5 millicuries Obtained through Life Sciences

Iodogen iodination procedures

Iodination takes place in the vial that contains the iodogen

1. Tap the vial to ensure all GH is at the bottom.
2. Add the GH to the iodogen vial
3. Add 20μl 0.5M PO_4.
4. Add 0.6mCi (6μl) I^{125}. Deglove.
5. Mix with gentle agitation.
6. Incubate for 15 minutes mixing gently at 5 minute intervals.
7. After 15 mins transfer the contents of the iodogen vial to a separate vial containing 200μl of 0.05M PO_4 buffer.

8. Add 200μl of Elution Buffer.

9. Gently mix well.

10. Transfer a 10μl aliquot to a specific activity (SA) tube and add 490μl elution buffer.

11. Add the remainder of iodinated protein to the column and elute in 1.0ml fractions per 20 minutes. Run the column overnight collecting approximately 80 fractions.

12. In the first 2 hours check to see the column is stable and the tubes on the fraction collector are aligned and moving as they should be.

Iodination of bovine TSH using Chloromine T

Chloromine T is an oxidising agent capable of converting iodide to a more reactive form. The procedure is simple requiring only mixing of solutions of the protein, sodium iodide (I125) and chloromine T. The reaction is terminated by the addition of a reducing agent, sodium metabisulphite.

Iodination components

PO_4 Stock Solution (0.5M PO_4)

7.80g $NaH_2PO_4 .2H_2O$ in 100 mls distilled water. Name 'A'.

35.51g Na_2HPO_4 anhydrous in 500 ml distilled water. Name 'B'.

(or 44.5g $Na_2HPO_4 . 2H_2O$)

Add 82mls of 'A' to the 500mls of 'B' , adjust to pH 7.4

Store as 40ml aliquots in screw top jars at -20°C.

This becomes the PO_4 stock solution.

0.05 M PO_4

Take PO_4 stock solution and dilute 1:10.

Make up 100mls for iodination.

Prepare fresh on the day of Iodination. Do not add azide.

Elution Buffer (200mls)

20mls PO_4 stock solution

0.4g BSA

0.2g NaN3

Adjust pH to 7.4

Make up to 200mls

Chloramine T

16mg Chloramine T

10mls 0.05M PO_4 buffer

Make up immediately prior to use.

Sodium Metabisulphite

16mg sodium metabisulphite

10mls 0.05M PO_4 buffer

Make up immediately prior to use.

Precipitation Buffer

2mls of Stock PO_4

1.861g EDTA (disodium,dihydrate)

1g BSA

0.1g Sodium Azide

Make up to 100mls with Milli Q water

Label (I^{125})

0.5 millicuries   Obtained through Life Sciences

Iodination of TSH

Iodination takes place in the vial that the TSH is stored.

1. Tap the vial to ensure all TSH is at the bottom.
2. Add 10µl 0.5M PO_4.
3. Add 0.5mCi (5µl) I^{125}. Deglove.
4. Mix with gentle agitation.
5. Add 5µl of Chloromine T.
6. Gently agitate for 60 seconds (Prepare for addition of sodium metabisulphate during this 60 seconds).
7. Add 10µl of Metabisulphate.
8. Agitate to mix well.
9. Add 500µl of elution buffer. Mix well.
10. Transfer a 10µl aliquot to a specific activity (SA) tube and add 490µl elution buffer.
11. Add the remainder of iodinated protein to the column and elute in 1.0ml fractions per 20 minutes. Run the column overnight collecting approximately 80 fractions.
12. In the first 2 hours check to see the column is stable and the tubes on the fraction collector are aligned and moving as they should be.

Appendix D: Significance tables

Table 9.1 Significance levels of fixed effects on responses to a thyrotropin-releasing hormone challenge.

	Time (min) before or after a TRH challenge at time zero													
	-20	-10	-5	2	5	10	15	20	30	45	60	90	120	150
TSH response[1]														
Year[2]			***[6]	***	**	***	**	***	***	***	***	***	***	
Grp(year)[3]			***	*	***	***	***	***	***	***	***	***	***	
Sire[4]			NS	***	***	***	***	***	***	***	***	***	***	
MGS[5]			NS	NS	NS	*	***	NS	**	***	**	***	***	
GH response[7]														
Year			***	***	***	NS	***	***	***	***	***			
Grp(year)			**	NS	***	***	***	***	***	***	***			
Sire			NS	NS	***	***	***	***	**	***	NS			
MGS			NS	NS	NS	***	**	NS	**	*	NS			
Prolactin response														
Year			NS	NS	NS	***	***	***	***		*		NS	NS
Grp(year)			***	*	***	***	***	***	***		***		***	***
Sire			***	NS	***	***	***	***	***		***		***	***
MGS			*	NS	NS	**	NS	NS	NS		NS		NS	NS
NEFA response[8]														
Year	NS	NS	*	NS	***	***	***	***	***	***	**			
Grp(year)	***	***	***	***	***	***	***	***	***	***	***			
Sire	***	NS	NS	NS	NS	NS	NS	NS	NS	**	*			
MGS	NS	NS	NS	*	*	**	NS	***	*	NS	*			
IGF-I response[9]														
Year	***									***				
Grp(year)	***									***				
Sire	***									***				
MGS	***									***				

[1] TSH – thyroid stimulating hormone

[2] year in which the heifer underwent the endocrine challenges

[3] challenge group nested within year

[4] each heifer was the offspring of one of six sires

[5] MGS – maternal grandsire, grouped within breed if there were 5 or less F^2 heifers

[6] * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS non-significant

[7] GH – growth hormone

[8] NEFA – non-esterified fatty acids

[9] IGF-I - insulin like growth factor 1

Table 9.2 Significance levels of fixed effects on responses to an adrenaline challenge.

	Time (min) before or after an adrenaline challenge at time zero										
	-20	**-10**	**-5**	**4**	**10**	**15**	**20**	**30**	**60**	**90**	**120**
NEFA response[1]											
Year[2]	***[6]	***	***	*	NS	*	NS	***	***		
Grp(year)[3]	***	***	***	***	***	***	***	***	***		
Sire[4]	**	*	NS	***	*	NS	NS	NS	**		
MGS[5]	*	**	**	*	***	*	*	NS	*		
Glycerol response											
Year	NS	NS	***	***	NS	*	***	***	***	**	
Grp(year)	***	***	***	***	***	***	***	***	***	***	
Sire	***	***	NS	**	*	***	**	***	***	***	
MGS	NS	NS	NS	*	NS	NS	**	NS	NS	NS	
Glucose response											
Year			NS	NS	NS	***	**	NS	NS	NS	NS
Grp(year)			***	***	***	***	***	***	***	***	***
Sire			NS	NS	NS	NS	NS	NS	NS	NS	NS
MGS			NS	NS	NS	NS	NS	NS	NS	NS	NS

[1] NEFA – non-esterified fatty acids

[2] year in which the heifer underwent the endocrine challenges

[3] challenge group nested within year

[4] each heifer was the offspring of one of six sires

[5] MGS – maternal grandsire, grouped within breed if there were 5 or less F^2 heifers

[6] * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS non-significant

Appendix E: Metabolic Phenotypes

Adrenaline challenge

Table 9.3 Methods used to calculate metabolic phenotypes from adrenaline challenge response data

Phenotype	Calculation
Glucose	
glu_max	Maximum glucose concentration
glu_t_max	Time at which maximum glucose concentration occurred
glu_bs	Baseline concentration – pooled sample of the -20, -10 and -5minutesamples taken prior to adrenaline infusion
glu_pk_20	The mean of the time at which maximum glucose concentration occurred was 18.59 the closest time a measurement occurred was at 20 minutes so this concentration was taken as the peak
glu_slope	Glucose – slope of the line calculated using Proc Reg calculated from 20 to 60 min
glu_AUC	1. Subtract baseline concentration from other adjusted concentrations (residual + mean). 2. Then calculate the area between consecutive time points by adding the baseline adjusted concentrations together, dividing this by two and then multiplying by the time difference between the two time points, e.g. av1=((bsadj4+bsadj10)/2)*(time_10-time_4) 3. Sum all the consecutive areas to give the total area 4. In the case of glu_auc, it was calculated from 4 to 90 minutes post infusion
Glycerol	
GLY_max	Maximum glycerol concentration
GLY_t_max	Time at which maximum glycerol concentration occurred
GLY_bs	Mean of the -20 and -10 glycerol concentrations
GLY_pk_4	The mean of the time at which maximum glycerol concentration occurred was 7.44 because this was in the middle between two actual measurement times both the 4 minute and 10 minute concentrations were designated as peak phenotypes
GLY_pk_10	As above
GLY_slope	Glycerol – slope of the line calculated using Proc Reg calculated from 20 to 60 min
GLY_RATE	GLY_RATE=(r_m_15-r_m_60)/(time_60-time_15)
GLY_auc4_30	Glycerol area under the curve calculated using the same method as for EEP_glu_auc but between 4 and 30 minutes
GLY_auc4_15	Glycerol area under the curve calculated using the same method as for EEP_glu_auc but between 4 and 15 minutes
NEFA	
N_max	Maximum NEFA concentration
N_t_max	Time at which maximum NEFA concentration occurred
N_bs	Baseline concentration – pooled sample of the -20, -10minutesamples taken prior to adrenaline infusion
N_pk_15	The mean of the time at which maximum NEFA concentration occurred was 15.4 the closest time a blood sample occurred was at 15 minutes so this concentration was taken as the peak

Table 9.3 Continued.

Phenotype	Calculation
N_slope	NEFA – slope of the line calculated using Proc Reg calculated from 20 to 60 min
N_auc	NEFA area under the curve calculated using the same method as for glu_auc but between 4 and 30 minutes
N_RATE	(r_m_20-r_m_60)/(time_60-time_20)
i_N_RATE	(r_m_m10-r_m_20)/30

Glucose challenge

Table 9.4 Methods used to calculate metabolic phenotypes from glucose challenge response data

Phenotype	Calculation
Glucose	
g_base	Glucose baseline (pooled sample of three samples taken at -20, -10 and -5 minutes prior to glucose infusion) = adjusted glucose concentration (residual (calculated from the model discussed earlier *) + mean (mean of all animals in the trial, 4.3))
g_k (%/min)	Fractional turnover rate of glucose (k) (Kaneko 1997) $k = \frac{\ln 1 - \ln 2}{T_2 - T_1} \times 100 = \% / \min$ where ln 1 = adjusted glucose concentration at 5 minutes; ln 2 = adjusted glucose concentration at 60 minutes; T_1 = time 5 minute sample was taken; T_2 = time 60 minute sample was taken
g_T_half (min)	Glucose clearance half-time (Kaneko 1997): $(0.693 / k) \times 100$
g_AUC_60min	1. Subtract baseline (-5) value from other adjusted values (residual + mean). 2. Then calculate the area between consecutive time points by adding the baseline adjusted concentrations together, dividing this by two and then multiplying by the time difference between the two time points, e.g. av1=((bsadj2+bsadj5)/2)*(time_5-time_2) 3. Sum all the consecutive areas to give the total area 4. In the case of g_Totarea_60min, it was calculated from 2 to 60 minutes post infusion
G_time	Glucose – slope of the line using Proc Reg calculated from 5 to 45 min
Insulin	
i_base	Insulin baseline (pooled sample of three samples taken at -20, -10 and -5 minutes prior to glucose infusion) = adjusted insulin concentration (residual (calculated from the model discussed earlier *) + mean (mean of all animals in the trial, 2.8))
i_AUC_45min	Insulin area under the curve calculated using the same method as for g_Totarea_60min but between 2 and 45 minutes
i_AUC_15min	Insulin area under the curve calculated using the same method as for g_Totarea_60min but between 2 and 15 minutes (acute AUC)
i_pk_10and15	Insulin peak concentration calculated as the mean of the adjusted (residual + mean) 10 and 15 minute concentrations
i_pk_10and15_bsadj	Insulin peak concentration calculated as the mean of the adjusted (residual + mean) 10 and 15 minute concentrations
i_15min	15 minute insulin concentration (residual + mean)
i_15min_bsadj	Baseline adjusted 15 minute insulin concentration = (residual + mean) – baseline
i_RATE	(i_pk_10and15 – ((r_m_45 + r_m_60)/2) / (mean of time 45 and 60) – (mean of time 10 and 15)
i_max	Maximum insulin concentration
i_t_max	Time maximum insulin concentration occurred
i_time	Insulin – slope of the line using Proc Reg calculated from 5 to 45 min

Table 9.4 Continued.

Phenotype	Calculation
ii_time	Insulin increase from baseline to peak – slope of the line calculated using Proc Reg from -5 to 10 min
expi_time	Insulin slope of the line from peak to baseline using Proc Reg, model: glu = time exptime (exptime = exp(-0.05*time))
NEFA	
N_min	Minimum NEFA concentration
N_t_min	Time minimum NEFA concentration occurred
N_time	NEFA – slope of the line using Proc Reg calculated from 5 to 45 min

TRH challenge

Table 9.5 Methods used to calculate metabolic phenotypes from TRH challenge response data

Phenotype	Calculation
Growth Hormone	
GH_AUC	1. Subtract baseline (-5) value from other adjusted values (residual + mean). 2. Then calculate the area between consecutive time points by adding the baseline adjusted concentrations together, dividing this by two and then multiplying by the time difference between the two time points, e.g. av1=((bsadj2+bsadj5)/2)*(time_5-time_2) 3. Sum all the consecutive areas to give the total area 4. In the case of GH_auc_total, it was calculated from 2 to 45 minutes post infusion
GH_auc15	Area under the curve between 2 and 15minutespost infusion calculated as shown for GH_auc_total
GH_auc15_30	Area under the curve between 15 and 30minutespost infusion calculated as shown for GH_auc_total
GHpk_mean	GH peak – calculated as the mean of the adjusted concentrations (residual + mean) at 5, 10, and 15 minutes post infusion, e.g. (r_m_5+r_m_10+r_m_15)/3
GHpk_mean_bsadj	GH peak – calculated as the mean of the 5, 10, and 15 minutes post-infusion adjusted concentrations (residual + mean) that were also adjusted for baseline, e.g. (bsadj5+bsadj10+bsadj15)/3
GH_RATE	Subtract the adjusted concentration (residual + mean) at 30minutesfrom the adjusted concentration at 15 minutes post-infusion and then divide this calculated value by the difference in time between 30 and 15 minutes, e.g. (r_m_15-r_m_30)/(time_30-time_15)
GH_max	Maximum concentration of GH (residual + mean)
GH_t_max	Time at which GH_max occurred
GH_bs	Baseline GH – Adjusted concentration (residual + mean) of the pooled pre-infusion samples, known as the -5 sample
pk_GH_5	Peak GH – Concentration of GH at the 5 minute sample time (residual + mean)
pk_GH_10	Peak GH – Concentration of GH at the 10 minute sample time (residual + mean)
GH_slope	Slope of the GH curve between 15 and 45 minutes calculated using proc reg with the model rslt = time and using adjusted data (residual + mean)
GH_slope2	Slope of the GH curve between 15 and 45 minutes calculated using proc reg with the model rslt = time time^2 and using adjusted data (residual + mean) using the output for time
GH_power2	Slope of the GH curve between 15 and 45 minutes calculated using proc reg with the model rslt = time time^2 and using adjusted data (residual + mean) using the output for time^2
IGF-I	
IGF-I_base	IGF-1 mean – calculated as the mean of the adjusted (residual + mean) IGF-1 concentrations, e.g. (r_m_m20+r_m_45)/2
NEFA	
n_AUC	Area under the NEFA response curve between -10 and 45minutesrelative to infusion calculated as shown for GH_AUC
n_auc15	Area under the NEFA response curve between -5 and 15minutesrelative to infusion calculated as shown for GH_AUC

Table 9.5 Continued.

Phenotype	Calculation
n_auc15_45	Area under the NEFA response curve between 15 and 45 minutes post infusion calculated as shown for GH_AUC
n_pk_mean	NEFA peak – calculated as the mean of the adjusted concentrations (residual + mean) at 5, 10, and 15 minutes post infusion, e.g. (r_m_5+r_m_10+r_m_15)/3
n_pk_mean_bsadj	NEFA peak – calculated as the mean of the adjusted concentrations (residual + mean) at 5, 10, and 15 minutes post infusion these values were also adjusted for baseline, e.g. (bsadj5+bsadj10+bsadj15)/3
n_RATE	Subtract the adjusted concentration (residual + mean) at 45 minutes from the adjusted concentration at 20 minutes post-infusion and then divide this calculated value by the difference in time between 45 and 20 minutes, e.g. (r_m_20-r_m_45)/(time_45-time_20)
NEFA_max	Maximum concentration of NEFA (residual + mean)
NEFA_t_max	Time at which NEFA_max occurred
NEFA_bs	Baseline NEFA – Concentration of NEFA (residual + mean)at -20 minutes relative to infusion
pk_NEFA_10	Peak NEFA – Concentration of NEFA at 10minutesample time (residual + mean)
n_slope	Slope of the decreasing part of the NEFA curve between 15 and 45 minutes calculated using proc reg with the model rslt = time $time^2$ and using adjusted data (residual + mean) using the output for time
n_slope2	Slope of the decreasing part of the NEFA curve between 15 and 45 minutes calculated using proc reg with the model rslt = time $time^2$ and using adjusted data (residual + mean) using the output for $time^2$
n_i_slope	Slope of the increasing part of the NEFA curve between -10 and 5 minutes calculated using proc reg with the model rslt = time and using adjusted data (residual + mean)
n_i_power2	Slope of the increasing part of the NEFA curve between -10 and 5 minutes calculated using proc reg with the model rslt = time $time^2$ and using adjusted data (residual + mean) using the output for time
n_i_slope2	Slope of the increasing part of the NEFA curve between -10 and 5 minutes calculated using proc reg with the model rslt = time $time^2$ and using adjusted data (residual + mean) using the output for $time^2$
Prolactin	
p_AUC	Area under the prolactin response curve between 2 and 60 minutes post infusion calculated as shown for GH_AUC
p_auc15	Area under the prolactin response curve between 2 and 15 minutes post-infusion calculated as shown for GH_AUC
p_auc15_30	Area under the prolactin response curve between 15 and 30 minutes post infusion calculated as shown for GH_AUC
p_RATE	Subtract the adjusted concentration (residual + mean) at 60 from the adjusted concentration at 20 minutes post-infusion and then divide this calculated value by the difference in time between 60 and 20 minutes, e.g. (r_m_20-r_m_60)/(time_60-time_20)
Prlc_max	Maximum concentration of prolactin (residual + mean)
Prlc_t_max	Time at which Prlc_max occurred
Prlc_bs	Baseline prolactin – concentration of Prlc
Pk_prlc_5	Peak prolactin – Concentration of Prlc at the 5 minute sample time (residual + mean)

Table 9.5 Continued.

Phenotype	Calculation
p_slope	Slope of the prolactin curve between 20 and 60 minutes calculated using proc reg with the model rslt = time and using adjusted data (residual + mean)
TSH	
t_AUC	Area under the TSH response curve between 2 and 90 minutes post infusion calculated as shown for GH_AUC
t_auc15	Area under the TSH response curve between 2 and 15 minutes post infusion calculated as shown for GH_ AUC
t_auc15_45	Area under the TSH response curve between 15 and 45 minutes post infusion calculated as shown for GH_ AUC auc_total
t_auc30	Area under the TSH response curve between 2 and 30 minutes post infusion calculated as shown for GH_ AUC
t_pk_mean	TSH peak – calculated as the mean of the adjusted concentrations (residual + mean) at 10, 15 and 20 minutes post infusion, e.g. (r_m_10+r_m_15+r_m_20)/3
t_pk_mean_bsadj	TSH peak – calculated as the mean of the adjusted concentrations (residual + mean) at 10, 15 and 20 minutes post infusion these values were also adjusted for baseline, e.g. (bsadj10+bsadj15+bsadj20)/3
t_RATE	Subtract the adjusted concentration (residual + mean) at 90 minutes from the adjusted concentration at 30 minutes post-infusion and then divide this calculated value by the difference in time between 90 and 30 minutes, e.g. (r_m_30-r_m_90)/(time_90-time_30)
TSH_max	Maximum concentration of TSH (residual + mean)
TSH_t_max	Time at which TSH_max occurred
TSH_bs	Baseline TSH – concentration of TSH
pk_TSH_15	Peak TSH – Concentration of TSH at the 15 minute sample time (residual + mean)
t_slope	Slope of the TSH curve between 20 and 90 minutes calculated using proc reg with the model rslt = time and using adjusted data (residual + mean)
t_slope2	Slope of the TSH curve between 20 and 90 minutes calculated using proc reg with the model rslt = time time^2 and using adjusted data (residual + mean) using the output for time
t_power2	Slope of the TSH curve between 20 and 90 minutes calculated using proc reg with the model rslt = time time^2 and using adjusted data (residual + mean) using the output for time^2

Appendix F: QTL locations

Table 9.6 The best predicted location (cM) for the metabolic (9.5 Appendix E) and milk production QTL (Table 9.7) as determined by the analysis models additive (A), additive + dominance (A+D), or half-sb (HS) together with the level of significance (Pval) the maximum F-value (Fval) and the 95% confidence interval (95%CI) of the QTL location.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
TRH	HS	p_auc15_30	1	98	0.0023	4.38	44 - 115
TRH	HS	p_auc_total	1	98	0.0076	3.93	35 - 123
Milk	HS	d60_pfy	2	46	0.0088	3.67	29 - 114
TRH	A	n_pk_mean	2	114	0.0026	6.2	18 - 125
TRH	A	pk_NEFA_10	2	114	0.0063	5.26	20 - 128
EEP	A+D	EEP_N_slope	2	131	0.0003	11.13	15 - 131
EEP	A+D	EEP_N_RATE	2	131	0.0029	8.39	8 - 131
EEP	HS	EEP_N_RATE	2	131	0.0031	4.17	3 - 131
EEP	HS	EEP_N_slope	2	131	0.0047	3.99	0 - 131
TRH	HS	t_auc15_45	3	68	0.0041	4.4	10 - 111
TRH	HS	t_auc_total	3	68	0.0072	3.92	13 - 112
TRH	HS	t_RATE	3	71	0.0064	3.86	10 - 102
TRH	HS	t_slope	3	72	0.0012	5.31	10 - 100
TRH	A	t_auc15_45	3	73	0.0045	5.71	49 - 124
TRH	A	t_slope	3	73	0.0059	5.65	44 - 123
TRH	HS	p_auc15_30	3	76	0.0059	3.8	2 - 95
TRH	A	t_auc_total	3	77	0.0037	5.84	45 - 124
TRH	A	t_pk_mean	3	77	0.0071	5.31	38 - 121
TRH	A	t_auc30	3	78	0.0051	5.54	39 - 124
TRH	A	t_pk_mean_bsadj	3	78	0.0093	5.13	0 - 122
TRH	A+D	t_auc15_45	3	79	0.0004	9.78	49 - 124
TRH	A+D	t_auc_total	3	80	0.0003	10.35	45 - 124
TRH	A+D	t_auc30	3	80	0.0015	8.91	39 - 124
TRH	A+D	t_pk_mean	3	80	0.0019	8.51	38 - 121
TRH	A+D	t_pk_mean_bsadj	3	80	0.0026	8.24	0 - 122
TRH	HS	t_slope2	3	86	0.0047	4.15	0 - 97
EEP	A+D	EPI_N_bs	3	97	0.0059	7.37	61 - 124
TRH	A	t_power2	3	113	0.0036	6.07	0 - 123
TRH	A+D	t_slope	3	113	0.0052	7.84	44 - 123
TRH	A+D	t_power2	3	114	0.0001	12.49	0 - 123
TRH	A+D	t_slope2	3	115	0.0006	9.43	0 - 120

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
GLU	A+D	N_min	3	123	0.0053	7.13	23 - 124
EEP	A	EPI_N_bs	3	124	0.0036	6.13	61 - 124
TRH	A	p_auc15	4	26	0.01	5.11	11 - 121
TRH	A	p_auc15_30	4	40	0.0059	5.72	16 - 83
TRH	HS	g_slope	4	53	0.0097	3.64	0 - 106
TRH	A	p_auc_total	4	76	0.0016	6.92	14 - 88
TRH	A+D	p_auc_total	4	79	0.0067	7.45	14 - 88
EEP	HS	GLY_t_max	4	83	0.0076	3.69	30 - 102
TRH	A+D	Pk_prlc_5	4	91	0.0024	8.47	0 - 121
TRH	A+D	p_auc15	4	91	0.0046	7.78	11 - 121
TRH	A+D	Emax_Prlc	4	91	0.0068	7.37	7 - 121
TRH	A	p_slope	5	64	0.0066	5.18	0 - 106
EEP	HS	EPI_N_t_max	5	83	0.0028	4.35	9 - 95
TRH	A	n_pk_mean	5	88	0.0046	5.61	18 - 106
EEP	HS	GLY_Intrcpt	5	92	0.0015	4.52	17 - 99
EEP	HS	GLY_auc15_60	5	92	0.0071	3.82	16 - 106
EEP	HS	GLY_slope	5	94	0.0012	4.73	17 - 105
Milk	HS	d60_pmsy	5	95	0.0093	3.71	0 - 106
Milk	HS	d60_pmsy	5	95	0.0093	3.71	0 - 106
Milk	HS	tot_pfy	5	96	0.0051	3.91	9 - 106
Milk	HS	tot_pcy	5	97	0.002	4.4	9 - 106
Milk	HS	tot_ptpy	5	98	0.0014	4.62	9 - 106
Milk	HS	tot_ptpy	5	98	0.0014	4.62	9 - 106
Milk	HS	tot_pcpy	5	98	0.0018	4.47	9 - 106
Milk	HS	d60_pwpy	5	98	0.0069	3.81	0 - 106
Milk	HS	d60_pcpy	5	100	0.0033	4.06	0 - 106
Milk	HS	d120_cppct	5	102	0	5.17	20 - 106
Milk	HS	d180_cppct	5	102	0	5.29	21 - 106
Milk	HS	tot_tppct	5	102	0	5.42	35 - 106
Milk	HS	d120_tppct	5	102	0	5.08	19 - 106
Milk	HS	d180_tppct	5	102	0	5.3	22 - 106
Milk	HS	d180_cpct	5	102	0	5.24	17 - 106
Milk	HS	d240_tppct	5	102	0.0001	5.05	20 - 106
Milk	HS	d120_cpct	5	102	0.0001	5.07	16 - 106
Milk	HS	tot_cppct	5	102	0.0002	5.3	34 - 106

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
Milk	HS	d270_tppct	5	102	0.0002	4.82	19 - 106
Milk	HS	d240_cpct	5	102	0.0002	4.81	14 - 106
Milk	HS	d240_cppct	5	102	0.0004	4.89	16 - 106
Milk	HS	d270_cppct	5	102	0.0005	4.77	15 - 106
Milk	HS	d270_cpct	5	102	0.0006	4.62	12 - 106
Milk	A	d240_wppct	5	102	0.0023	5.88	22 - 106
Milk	A	d240_tppct	5	102	0.0027	6.31	9 - 106
Milk	A	d270_tppct	5	102	0.0027	6.04	6 - 106
Milk	A	d60_pcpy	5	102	0.0039	6.04	46 - 106
Milk	HS	d60_pcy	5	102	0.004	3.99	0 - 106
Milk	A	d60_pcy	5	102	0.0043	5.85	47 - 106
Milk	A	d270_wppct	5	102	0.0043	5.42	23 - 106
Milk	A	d240_cppct	5	102	0.0044	6.02	5 - 106
Milk	A	d180_wppct	5	102	0.0044	5.97	22 - 106
Milk	A	d270_cppct	5	102	0.0049	5.99	1 - 106
Milk	HS	d60_cpct	5	102	0.0051	3.87	7 - 106
Milk	A	d60_pwpy	5	102	0.006	5.62	46 - 106
Milk	A	d120_tppct	5	102	0.0098	5.39	15 - 106
Milk	A	d120_wppct	5	102	0.0098	4.76	20 - 106
Milk	HS	tot_cpct	5	103	0	5.21	22 - 106
Milk	A	tot_tppct	5	103	0.002	6.61	13 - 106
Milk	A	tot_wppct	5	103	0.0025	6.05	20 - 106
Milk	A+D	tot_cppct	5	103	0.0037	6.47	9 - 106
Milk	A	d180_tppct	5	103	0.0037	6.24	9 - 106
Milk	A	d180_cppct	5	103	0.0056	6.04	9 - 106
Milk	HS	tot_pwpy	5	104	0	5.32	20 - 106
Milk	A	tot_pcy	5	105	0.0084	5.65	9 - 106
Milk	A	tot_pwpy	5	106	0	9.2	70 - 106
Milk	A+D	tot_pwpy	5	106	0.0009	9.36	70 - 106
Milk	A	tot_ptpy	5	106	0.002	6.63	29 - 106
Milk	A	tot_pcpy	5	106	0.0032	6.38	12 - 106
Milk	A	tot_ptpy	5	106	0.0039	6.63	9 - 106
Milk	A+D	tot_ptpy	5	106	0.0077	7.07	9 - 106
Milk	A+D	tot_pcpy	5	106	0.0083	6.89	12 - 106
Milk	A+D	tot_ptpy	5	106	0.0091	7.07	29 - 106

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
GLU	A+D	t_auc15	6	34	0.0022	8.86	0 - 80
TRH	A+D	t_auc15	6	34	0.0022	8.86	0 - 80
GLU	A	g_k	6	65	0.0046	5.79	34 - 97
GLU	A	g_T_half	6	65	0.0093	5.31	34 - 101
GLU	A+D	g_k	6	67	0.006	7.23	34 - 97
Milk	A+D	d270_cpct	6	72	0.0093	7.88	10 - 101
Milk	HS	tpt_cpct	6	76	0.0004	5.11	40 - 88
Milk	HS	d270_cpct	6	76	0.0004	5.2	39 - 91
Milk	HS	d180_cpct	6	76	0.0006	5.21	44 - 87
Milk	HS	d240_cpct	6	77	0.0005	5.33	41 - 90
Milk	HS	d120_cpct	6	77	0.0006	4.86	44 - 86
Milk	HS	d60_cpct	6	78	0.0009	4.78	48 - 86
Milk	HS	d120_tppct	6	78	0.0077	3.86	37 - 89
Milk	A	d240_cpct	6	78	0.0083	6.61	10 - 98
Milk	A	tpt_cpct	6	78	0.0094	6.77	10 - 97
Milk	HS	d240_cppct	6	79	0.0022	4.42	34 - 92
Milk	HS	d270_cppct	6	79	0.0024	4.33	34 - 94
Milk	HS	d240_tppct	6	79	0.0024	4.28	34 - 91
Milk	HS	d180_cppct	6	79	0.0027	4.29	35 - 89
Milk	HS	d270_tppct	6	79	0.003	4.22	34 - 93
Milk	HS	tot_cppct	6	79	0.0038	4.17	29 - 91
Milk	HS	tot_tppct	6	79	0.004	4.12	30 - 90
Milk	HS	d120_cppct	6	79	0.0042	4.08	38 - 91
Milk	HS	d180_tppct	6	79	0.0042	4.07	35 - 91
Milk	HS	d60_cppct	6	79	0.0051	4.06	43 - 97
Milk	A	d270_cpct	6	87	0.0092	6.67	10 - 101
EEP	A	GLY_auc4_15	7	0	0.0095	4.95	0 - 91
EEP	A	EPI_N_max	7	34	0.0037	5.89	0 - 115
Milk	A	tot_lpct	7	35	0.0016	10.64	3 - 35
Milk	A	d240_lpct	7	35	0.0022	11.47	3 - 35
Milk	A	d270_lpct	7	35	0.0035	11.25	3 - 35
Milk	A+D	d240_lpct	7	35	0.0036	11.76	3 - 35
Milk	A+D	d270_lpct	7	35	0.0046	11.56	3 - 35
Milk	A+D	tot_lpct	7	35	0.0049	10.98	3 - 35
Milk	A	d180_lpct	7	35	0.0077	10.59	23 - 35

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
Milk	A+D	d180_lpct	7	35	0.0097	11.01	23 - 35
EEP	HS	GLY_pk_10	7	89	0.0033	4.17	13 - 107
GLU	HS	iAUC_gAUC	7	121	0.0018	4.84	3 - 121
GLU	HS	bsadj10i_basadj10g	7	121	0.0022	4.6	1 - 121
GLU	HS	expi_time	7	121	0.0028	4.46	2 - 121
GLU	HS	ii_time	7	121	0.0036	4.34	1 - 121
GLU	HS	ins_max	7	121	0.0048	4.32	1 - 121
GLU	HS	i_Totarea_45min	7	121	0.0057	4.2	2 - 121
GLU	HS	i_pk_10and15	7	121	0.0063	4.1	0 - 121
GLU	HS	i_pk_10and15_bsadj	7	121	0.0069	4.07	0 - 121
GLU	HS	i_15min_bsadj	7	121	0.0093	3.91	1 - 121
GLU	HS	i_15min	7	121	0.0095	3.97	0 - 121
TRH	A	p_slope	8	0	0.0079	5.3	0 - 98
TRH	A	Emax_Prlc	8	0	0.0084	5.36	0 - 89
TRH	A	p_RATE	8	0	0.0088	5.15	0 - 99
TRH	A	p_RATE_bsadj	8	0	0.009	5.11	0 - 97
Milk	A+D	d60_pwpy	8	10	0.0025	8.59	0 - 100
Milk	A+D	d60_pcpy	8	10	0.0048	7.7	0 - 102
Milk	A+D	d60_ptpy	8	10	0.0062	7.35	0 - 115
TRH	A+D	t_auc15_45	8	44	0.0095	6.87	34 - 117
EEP	A	glu_t_max	8	85	0.0019	6.28	59 - 106
EEP	A	EEP_glu_auc	8	91	0	11.94	82 - 101
EEP	A+D	EEP_glu_auc	8	92	0.0002	12.03	82 - 101
EEP	HS	glu_t_max	8	98	0.0033	4.03	0 - 115
EEP	A	EEP_glu_slope	8	110	0.0008	8.23	59 - 115
EEP	A+D	EEP_glu_slope	8	110	0.0028	8.29	59 - 115
EEP	A	EEP_glu_Intrcpt	8	111	0.006	5.52	48 - 115
TRH	A	t_pk_mean	8	112	0.0061	5.48	33 - 117
TRH	A+D	GHAUC15_igf	9	28	0.003	7.59	20 - 45
TRH	A	GHAUC15_igf	9	30	0.0007	7.49	20 - 45
GLU	A	I_time	9	31	0.0006	6.99	24 - 108
GLU	A	i_Rate_bsadj	9	31	0.0012	7.13	24 - 102
GLU	A	i_Rate	9	31	0.0014	6.9	24 - 96
TRH	A	GHpk_mean_bsadj	9	31	0.0016	6.56	20 - 64
GLU	A+D	i_Rate_bsadj	9	31	0.0063	7.14	24 - 102

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
GLU	A	bsadj20i_basadj20g	9	32	0.0002	8.59	19 - 89
GLU	A	ins_max	9	32	0.0004	9.2	24 - 100
TRH	A	GH_auc15	9	32	0.0021	7.05	24 - 57
GLU	A+D	I_time	9	32	0.0059	7.17	24 - 108
GLU	A+D	i_Rate	9	32	0.0088	6.92	24 - 96
GLU	A+D	ins_max	9	33	0.0006	9.31	24 - 100
GLU	A+D	bsadj20i_basadj20g	9	33	0.0016	8.71	19 - 89
TRH	A+D	GHpk_mean_bsadj	9	33	0.0084	6.67	20 - 64
GLU	A	i_pk_10and15_bsadj	9	34	0.0003	8.92	24 - 86
GLU	A	i_area15min	9	34	0.0004	7.93	24 - 82
GLU	A	expi_time	9	34	0.0007	7.76	24 - 93
TRH	A+D	GH_auc15	9	34	0.0059	7.11	24 - 57
GLU	A	i_pk_10and15	9	35	0.0002	8.82	24 - 92
GLU	A	i_15min_bsadj	9	35	0.0002	9.09	24 - 101
GLU	A+D	i_pk_10and15_bsadj	9	35	0.0015	9.01	24 - 86
GLU	A+D	i_area15min	9	35	0.0032	7.95	24 - 82
TRH	A	GHpk_mean	9	35	0.0061	5.41	19 - 104
TRH	A	pk_GH_10	9	35	0.0075	5.23	20 - 108
GLU	A	i_15min	9	36	0.0004	8.42	24 - 103
GLU	A+D	i_pk_10and15	9	36	0.0009	8.91	24 - 92
GLU	A+D	expi_time	9	36	0.002	8.37	24 - 93
TRH	A	igf_insAUC15	9	37	0	10.3	24 - 87
GLU	A	i_Totarea_45min	9	37	0.0001	10.04	24 - 64
GLU	A	iAUC_gAUC	9	37	0.0004	8.58	24 - 93
GLU	A+D	i_Totarea_45min	9	37	0.0005	10.1	24 - 64
GLU	A+D	i_15min_bsadj	9	37	0.001	9.28	24 - 101
GLU	A+D	iAUC_gAUC	9	37	0.0012	8.72	24 - 93
GLU	A	bsadj10i_basadj10g	9	37	0.0013	7.29	24 - 98
TRH	A+D	igf_insAUC15	9	38	0.0001	10.7	24 - 87
GLU	A	ii_time	9	38	0.0004	7.27	24 - 92
GLU	A+D	i_15min	9	38	0.0022	8.6	24 - 103
GLU	A+D	bsadj10i_basadj10g	9	38	0.0053	7.45	24 - 98
GLU	A+D	ii_time	9	38	0.0062	7.34	24 - 92
TRH	A	n_pk_mean	9	93	0.0051	5.71	37 - 104
TRH	A	t_pk_mean_bsadj	11	82	0.0045	5.73	36 - 93

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
TRH	A	t_pk_mean	11	82	0.0075	5.33	36 - 94
TRH	A	t_auc15_45	11	85	0.0085	5.09	36 - 104
TRH	A	t_auc_total	11	87	0.0004	8.71	53 - 98
TRH	A	t_auc30	11	87	0.0005	7.86	67 - 101
TRH	A+D	t_auc30	11	87	0.0006	9.2	67 - 101
TRH	A+D	t_auc_total	11	87	0.0009	9.56	53 - 98
TRH	A	t_auc15	11	88	0.0021	6.67	67 - 107
TRH	A+D	t_auc15	11	88	0.0053	7.61	67 - 107
Milk	HS	d120_wppct	11	109	0	6.22	0 - 118
Milk	HS	d60_wppct	11	110	0	6.99	3 - 118
Milk	HS	d240_wppct	11	110	0.0034	4.22	0 - 117
Milk	HS	d180_wppct	11	111	0.0002	5.38	0 - 117
Milk	HS	tot_wppct	11	111	0.0014	4.59	0 - 117
Milk	HS	d270_wppct	11	111	0.0067	3.94	0 - 117
Milk	HS	tot_pwpy	11	115	0.0002	5.28	29 - 118
TRH	A	Emax_NEFA	11	115	0.0037	6.01	17 - 118
TRH	A+D	pk_TSH_15	13	5	0.0009	9.2	1 - 86
TRH	A+D	t_auc15_45	13	6	0.0033	7.69	0 - 52
TRH	A+D	t_auc_total	13	7	0	11.01	0 - 34
TRH	A+D	t_pk_mean_bsadj	13	7	0.0003	10.42	1 - 34
TRH	A+D	t_pk_mean	13	7	0.0004	10.66	2 - 34
TRH	A+D	t_auc30	13	9	0.0006	9.37	0 - 82
TRH	A+D	Emax_TSH	13	10	0.0071	6.85	0 - 86
GLU	HS	N_time	13	12	0.0078	3.72	2 - 86
TRH	A+D	t_auc15	13	14	0.0004	10.46	1 - 85
TRH	A	pk_TSH_15	13	18	0.0011	6.86	1 - 86
TRH	A	t_auc30	13	20	0.0013	6.47	0 - 82
TRH	A	t_auc15	13	20	0.003	6.28	1 - 85
TRH	A	t_auc15_45	13	20	0.0038	5.59	0 - 52
TRH	A	t_auc_total	13	21	0.0003	7.86	0 - 34
TRH	A	t_pk_mean	13	23	0.0001	8.89	2 - 34
TRH	A	t_pk_mean_bsadj	13	23	0.0005	8.49	1 - 34
TRH	HS	t_pk_mean	13	25	0.0046	4.32	7 - 86
TRH	HS	t_pk_mean_bsadj	13	26	0.0064	4.2	5 - 86
TRH	HS	n_slope	13	35	0.0062	3.98	10 - 86

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
TRH	HS	n_RATE_bsadj	13	36	0.0026	4.05	31 - 86
TRH	HS	n_RATE	13	37	0.002	4.14	29 - 86
TRH	HS	GH_auc15_30	13	38	0.0069	3.72	12 - 86
TRH	HS	n_auc15	13	40	0.0038	3.97	3 - 85
TRH	A	GHpk_mean_bsadj	13	44	0.0082	4.88	0 - 62
TRH	HS	n_pk_mean_bsadj	13	57	0.0013	4.37	1 - 82
TRH	HS	pk_TSH_15	13	86	0.001	4.81	5 - 86
Milk	A	d270_fcpt	14	0	0	8.38	0 - 61
Milk	A	d240_mspct	14	0	0	9.15	0 - 62
Milk	A	d120_fpct	14	0	0.0001	8.9	0 - 58
Milk	A	d240_fcpt	14	0	0.0001	8.62	0 - 61
Milk	A	tot_mspct	14	0	0.0001	9.08	0 - 62
Milk	A	d180_mspct	14	0	0.0001	9.42	0 - 62
Milk	A	d240_mspct	14	0	0.0001	8.97	0 - 62
Milk	A	d60_fpct	14	0	0.0002	8.22	0 - 49
Milk	A	d180_fpct	14	0	0.0002	8.82	0 - 60
Milk	A	d120_mspct	14	0	0.0002	9.33	0 - 47
Milk	A	d60_mspct	14	0	0.0003	8.23	0 - 62
Milk	A+D	d120_fpct	14	0	0.0008	8.9	0 - 58
Milk	A+D	d120_mspct	14	0	0.0009	9.46	0 - 47
Milk	A+D	d180_fpct	14	0	0.0013	8.83	0 - 60
Milk	A+D	d180_mspct	14	0	0.0013	9.51	0 - 62
Milk	A+D	tot_mspct	14	0	0.0015	9.11	0 - 62
Milk	HS	d180_fpct	14	0	0.0016	4.86	0 - 63
Milk	A+D	d240_mspct	14	0	0.0018	9.21	0 - 62
Milk	A+D	d60_mspct	14	0	0.0019	8.44	0 - 62
Milk	A+D	d60_fpct	14	0	0.0021	8.28	0 - 49
Milk	A+D	d270_fcpt	14	0	0.0022	8.4	0 - 61
Milk	HS	d240_fcpt	14	0	0.0024	4.57	0 - 63
Milk	A+D	d240_mspct	14	0	0.0026	9	0 - 62
Milk	HS	d120_fpct	14	0	0.0027	4.64	0 - 63
Milk	A+D	d240_fcpt	14	0	0.0032	8.64	0 - 61
Milk	HS	d270_fcpt	14	0	0.0036	4.44	0 - 63
Milk	HS	d180_mspct	14	0	0.0053	4.05	0 - 63
Milk	HS	d120_mspct	14	0	0.0071	3.91	0 - 65

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
Milk	HS	tot_mspct	14	0	0.0087	3.84	0 - 63
Milk	HS	d240_mspct	14	0	0.0087	3.83	0 - 63
GLU	A	i_r_m_m5	14	38	0.0011	7.47	38 - 91
GLU	A+D	i_r_m_m5	14	38	0.0047	7.93	38 - 91
EEP	A+D	EEP_N_Intrcpt	14	41	0.01	6.94	0 - 95
EEP	A	EEP_N_Intrcpt	14	45	0.0093	5.1	0 - 95
TRH	A	igf_mean	14	47	0.0006	7.72	38 - 65
TRH	A+D	igf_mean	14	47	0.0043	7.73	38 - 65
Milk	A+D	d60_pmy	14	48	0	11.07	13 - 56
Milk	A+D	d60_ply	14	48	0.0001	11.18	21 - 56
Milk	A	d60_ply	14	49	0	10.37	21 - 56
Milk	A	d60_pmy	14	49	0.0001	10.09	13 - 56
Milk	A+D	d60_pcpy	14	51	0	11.72	37 - 55
Milk	A	d60_pcpy	14	51	0	11.31	37 - 55
Milk	A	d60_ptpy	14	51	0	11.26	38 - 54
Milk	A	d60_pcy	14	51	0.0001	11.14	35 - 56
Milk	A+D	d60_ptpy	14	51	0.0001	11.59	38 - 54
Milk	A+D	d60_pcy	14	51	0.0002	11.57	35 - 56
Milk	A	d60_pmsy	14	51	0.0002	8.72	22 - 60
EEP	A	GLY_Intrcpt	14	51	0.0012	7.27	47 - 91
Milk	A	d60_pwpy	14	51	0.0012	7.92	20 - 54
Milk	A+D	d60_pmsy	14	51	0.0016	9.53	22 - 60
Milk	A+D	d60_pwpy	14	51	0.0031	7.92	20 - 54
EEP	A+D	GLY_Intrcpt	14	51	0.0067	7.35	47 - 91
Milk	A	tot_pwpy	14	54	0.0044	5.84	7 - 76
Milk	A	tot_pcpy	14	56	0.004	5.64	12 - 95
Milk	A	tot_pmy	14	56	0.01	4.95	0 - 95
EEP	A	GLY_auc15_60	14	63	0.0049	5.98	45 - 95
EEP	A+D	GLY_auc15_60	14	65	0.0064	7.5	45 - 95
EEP	HS	GLY_pk_10	15	1	0.0023	4.14	0 - 68
EEP	A	glu_pk_20	15	2	0.0041	5.35	0 - 38
EEP	HS	GLY_bs	15	3	0.0065	3.72	0 - 76
GLU	HS	G_time	15	34	0.0001	5.65	22 - 52
GLU	HS	g_k	15	36	0.0001	5.07	22 - 51
GLU	HS	expi_time	15	37	0.0033	4.34	0 - 64

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
GLU	HS	ins_max	15	38	0.0005	5.4	13 - 69
GLU	HS	ii_time	15	38	0.0006	4.97	1 - 71
GLU	HS	g_T_half	15	38	0.0007	4.29	0 - 56
GLU	HS	I_time	15	38	0.0016	4.7	7 - 69
GLU	HS	i_Totarea_45min	15	46	0.0048	4.21	0 - 73
GLU	HS	iAUC_gAUC	15	47	0.0065	4.11	0 - 73
GLU	HS	i_area15min	15	48	0.001	5.03	10 - 73
GLU	HS	i_pk_10and15	15	48	0.0011	4.73	24 - 73
GLU	HS	i_15min	15	48	0.0026	4.3	13 - 71
GLU	HS	i_15min_bsadj	15	48	0.0029	4.52	25 - 72
GLU	HS	bsadj10i_basadj10g	15	48	0.0051	4.2	4 - 75
GLU	HS	i_Rate_bsadj	15	49	0.0005	5.33	22 - 73
GLU	HS	i_Rate	15	49	0.0006	5.21	26 - 73
EEP	HS	EEP_N_RSQ	15	49	0.0008	4.43	13 - 64
TRH	HS	igf_insAUC15	15	49	0.0009	4.7	13 - 72
GLU	HS	i_pk_10and15_bsadj	15	49	0.0012	4.93	27 - 72
EEP	A+D	glu_bs	16	0	0.00076	6.99	0 - 67
EEP	A	glu_bs	16	0	0.0099	5.13	0 - 67
EEP	HS	EEP_N_auc	16	7	0.0023	4.47	0 - 85
TRH	A	GH_bs	16	21	0.0021	6.76	2 - 73
TRH	A+D	GH_bs	16	21	0.0094	6.94	2 - 73
TRH	A	GHbs_igf	16	22	0.0044	6.03	4 - 48
TRH	HS	n_i_slope	16	41	0.0084	3.77	2 - 51
TRH	A	g_slope	17	25	0.0064	5.51	2 - 58
Milk	A	d60_lpct	17	51	0.0001	10.22	49 - 63
Milk	A	d120_lpct	17	51	0.0009	8.71	47 - 52
Milk	A+D	d60_lpct	17	51	0.0021	10.24	49 - 63
Milk	A	d180_lpct	17	51	0.0028	7.57	44 - 60
Milk	A	tot_lpct	17	51	0.0049	6.8	32 - 60
Milk	A+D	d120_lpct	17	51	0.0065	8.78	47 - 52
Milk	A	d240_lpct	17	51	0.0075	6.69	32 - 60
Milk	A+D	d180_lpct	17	51	0.0092	7.78	44 - 60
Milk	A	d120_cpct	17	53	0.0047	5.93	25 - 60
Milk	A	d240_cpct	17	53	0.005	5.62	8 - 60
Milk	A	d180_cpct	17	53	0.0055	5.87	10 - 60

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
Milk	A	d270_cpct	17	53	0.0093	5.26	8 - 60
Milk	A	d120_tppct	17	54	0.0045	5.9	20 - 66
Milk	A	d120_cppct	17	54	0.0052	5.61	25 - 79
Milk	A	tot_cpct	17	54	0.0054	5.86	8 - 60
Milk	A	d240_cppct	17	54	0.0083	5.12	8 - 65
Milk	A	tot_tppct	17	55	0.0039	5.63	8 - 80
Milk	A	d180_tppct	17	55	0.0052	5.73	8 - 72
Milk	A	d240_tppct	17	55	0.006	5.34	8 - 65
Milk	A	d180_cppct	17	55	0.0061	5.45	8 - 76
Milk	A	tot_cppct	17	55	0.0064	5.46	8 - 66
Milk	A	tot_mspct	17	57	0.0021	6.16	10 - 63
Milk	A	d240_mspct	17	57	0.0027	5.72	8 - 62
Milk	A	d240_mspct	17	57	0.0037	5.4	18 - 75
Milk	A	d120_mspct	17	58	0.0027	5.81	18 - 80
Milk	A	d180_mspct	17	58	0.0031	6.01	8 - 77
Milk	HS	tot_lpct	17	60	0.0012	4.81	32 - 68
Milk	HS	d240_lpct	17	60	0.0036	4.35	32 - 67
Milk	HS	d270_lpct	17	60	0.0052	4.13	19 - 68
Milk	HS	d180_lpct	17	61	0.0028	4.52	34 - 68
Milk	HS	d120_lpct	17	62	0.0032	4.38	32 - 68
Milk	HS	d60_lpct	17	63	0.0044	4.21	32 - 68
GLU	A	i_15min_bsadj	18	21	0.0018	5.99	5 - 62
GLU	A	i_pk_10and15	18	21	0.0023	6.22	5 - 44
GLU	A	i_Rate	18	21	0.0027	5.96	1 - 40
GLU	A	i_Rate_bsadj	18	21	0.0027	5.9	3 - 40
GLU	A	expi_time	18	21	0.0028	5.88	5 - 37
GLU	A	i_15min	18	21	0.0032	5.83	4 - 42
GLU	A	i_pk_10and15_bsadj	18	21	0.0034	5.94	5 - 74
GLU	A	bsadj10i_basadj10g	18	21	0.005	5.42	5 - 48
GLU	A	i_area15min	18	21	0.0061	5.05	2 - 48
GLU	A	iAUC_gAUC	18	21	0.0063	4.87	4 - 84
GLU	A	i_Totarea_45min	18	21	0.0083	5.02	1 - 74
GLU	A+D	expi_time	18	21	0.0096	5.99	5 - 37
GLU	A+D	i_pk_10and15	18	21	0.0098	6.23	5 - 44
GLU	HS	expi_time	18	29	0.0058	4.09	3 - 77

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
GLU	HS	i_15min	18	30	0.0059	4.18	15 - 91
GLU	HS	i_15min_bsadj	18	30	0.0065	4.12	16 - 82
GLU	HS	i_Rate	18	31	0.0089	3.99	20 - 88
GLU	HS	i_Rate_bsadj	18	31	0.0095	3.93	21 - 83
GLU	A	N_t_min	18	32	0.0085	4.73	14 - 74
GLU	HS	i_pk_10and15	18	32	0.0088	3.92	16 - 88
GLU	HS	i_pk_10and15_bsadj	18	33	0.01	3.89	16 - 86
GLU	HS	I_time	18	34	0.005	4.16	10 - 91
GLU	HS	bsadj10i_basadj10g	18	34	0.0067	4.04	21 - 91
TRH	HS	Emax_TSH	19	38	0.0066	4.18	0 - 68
TRH	HS	igf_mean	19	41	0.0068	3.96	2 - 83
Milk	A	tot_pmy	19	59	0.0008	7.42	26 - 83
Milk	A	tot_pmsy	19	59	0.0017	6.83	25 - 63
Milk	A+D	tot_pmy	19	59	0.0034	7.45	26 - 83
Milk	A	tot_pfy	19	59	0.0086	5.38	20 - 63
Milk	A+D	tot_pmsy	19	59	0.0089	6.83	25 - 63
Milk	A	tot_ply	19	60	0.0009	7.02	24 - 83
Milk	A	tot_ply	19	60	0.0012	7.02	26 - 83
Milk	A+D	tot_ply	19	60	0.0053	7.03	26 - 83
Milk	A+D	tot_ply	19	60	0.0059	7.03	24 - 83
Milk	A	tot_ptpy	19	60	0.0083	4.9	20 - 63
Milk	A+D	d270_wppct	19	78	0.009	6.32	27 - 100
Milk	A+D	tot_wppct	19	79	0.007	6.89	29 - 102
Milk	A+D	d240_cppct	19	79	0.008	7.18	36 - 97
Milk	A+D	d180_wppct	19	80	0.0027	7.67	54 - 103
Milk	A	d270_cppct	19	80	0.006	6.31	11 - 97
Milk	A	d270_wppct	19	80	0.0064	5.38	27 - 100
Milk	A+D	d180_tppct	19	80	0.0066	7.64	68 - 97
Milk	A+D	d180_cppct	19	80	0.0073	7.5	56 - 98
Milk	A	d240_tppct	19	81	0.0033	6.7	31 - 97
Milk	A	d240_cppct	19	81	0.0039	6.71	36 - 97
Milk	A	tot_cppct	19	81	0.0077	6.09	20 - 99
Milk	A	tot_tppct	19	81	0.0077	6.17	34 - 100
Milk	A	d270_tppct	19	81	0.0081	6.18	26 - 96
Milk	A	tot_wppct	19	82	0.003	6.24	29 - 102

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
Milk	A	d180_tppct	19	82	0.0032	7.07	68 - 97
Milk	A	d180_cppct	19	82	0.0045	7.03	56 - 98
Milk	A	d240_wppct	19	82	0.0053	5.48	38 - 101
Milk	A+D	d120_tppct	19	82	0.0075	7.5	68 - 99
Milk	A	d180_wppct	19	83	0.0028	6.51	54 - 103
Milk	A	d120_cppct	19	83	0.0048	6.66	57 - 98
Milk	A	d120_tppct	19	83	0.0051	6.77	68 - 99
Milk	A	d120_wppct	19	83	0.0058	5.21	35 - 107
Milk	A+D	d120_wppct	19	83	0.0092	6.44	35 - 107
TRH	A	n_RATE	19	108	0.0005	7.26	68 - 111
TRH	A	n_RATE_bsadj	19	108	0.0011	7.32	70 - 111
TRH	A+D	n_RATE_bsadj	19	111	0.0037	7.6	70 - 111
TRH	A+D	n_RATE	19	111	0.0062	7.5	68 - 111
EEP	A	EEP_glu_slope	20	26	0.0012	7.2	16 - 39
EEP	A+D	EEP_glu_slope	20	26	0.0044	7.25	16 - 39
Milk	HS	d270_lpct	20	41	0.0047	3.82	0 - 68
Milk	HS	d240_lpct	20	42	0.0057	3.77	0 - 68
Milk	HS	tot_lpct	20	43	0.0063	3.73	0 - 68
Milk	A	d180_cpct	20	57	0.008	4.8	3 - 68
Milk	HS	d180_lpct	20	58	0.0081	3.67	0 - 68
Milk	A+D	d240_cpct	20	59	0.0039	5.49	16 - 68
Milk	A	d270_cpct	20	60	0.0018	5.78	39 - 68
Milk	A	d180_cppct	20	60	0.0089	4.85	13 - 68
Milk	A	d240_cppct	20	61	0.0032	5.69	17 - 68
Milk	A	tot_cppct	20	61	0.0093	4.57	8 - 68
Milk	A+D	d270_cppct	20	62	0.0038	5.91	48 - 68
Milk	A	d240_tppct	20	62	0.0046	5.34	27 - 68
Milk	A	d270_tppct	20	63	0.0038	5.57	21 - 68
Milk	A	d270_cppct	20	65	0.01	6.04	48 - 68
Milk	HS	d60_mspct	21	0	0.0004	4.61	0 - 53
Milk	HS	d120_mspct	21	0	0.0011	4.39	0 - 53
Milk	HS	d180_mspct	21	0	0.0047	3.86	0 - 53
Milk	HS	d60_fpct	21	0	0.0063	3.82	0 - 92
TRH	A	pk_NEFA_10	22	16	0.0084	4.69	0 - 63
GLU	A	g_r_m_m5	22	66	0.0027	5.43	30 - 66

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
GLU	A+D	g_r_m_m5	22	66	0.0088	6.41	30 - 66
GLU	HS	i_Totarea_45min	23	21	0.009	3.98	0 - 69
EEP	HS	EEP_N_RATE	23	30	0.0031	4.13	26 - 69
EEP	HS	EEP_N_slope	23	30	0.0093	3.64	23 - 69
Milk	HS	d240_cpct	23	36	0.0063	3.74	4 - 59
Milk	HS	d270_cpct	23	36	0.0071	3.73	6 - 59
TRH	A+D	g_slope	23	37	0.0039	7.67	5 - 66
Milk	HS	tot_cpct	23	37	0.0048	3.81	4 - 59
Milk	HS	d180_cpct	23	37	0.0066	3.75	0 - 59
TRH	A	GH_RATE_bsadj	23	38	0.0057	5.74	18 - 66
TRH	A	g_slope	23	39	0.0011	7.26	5 - 66
TRH	A	Emax_GH	23	42	0.0074	5.33	0 - 47
Milk	HS	d120_cpct	23	43	0.0098	3.56	0 - 59
Milk	A	d60_pwpy	23	59	0.0075	5.24	28 - 68
GLU	A	I_time	25	0	0.0012	6.9	0 - 44
GLU	A	expi_time	25	0	0.0016	6.46	0 - 40
GLU	A	i_15min_bsadj	25	0	0.0018	6.06	0 - 51
GLU	A	i_pk_10and15	25	0	0.0019	6.15	0 - 27
GLU	A	i_15min	25	0	0.0021	6.2	0 - 29
GLU	A	i_pk_10and15_bsadj	25	0	0.0026	5.7	0 - 28
GLU	A	i_Rate	25	0	0.0026	6.09	0 - 33
GLU	A	i_Rate_bsadj	25	0	0.0027	5.97	0 - 26
GLU	A+D	I_time	25	0	0.0043	7.12	0 - 44
GLU	A+D	i_Rate	25	0	0.0053	6.82	0 - 33
GLU	A	bsadj10i_basadj10g	25	0	0.0054	5.11	0 - 40
GLU	A	i_area15min	25	0	0.0058	5.04	0 - 33
GLU	A	ins_max	25	0	0.0058	5.03	0 - 48
GLU	A+D	i_pk_10and15	25	0	0.007	6.78	0 - 27
GLU	A	ii_time	25	0	0.0075	4.84	0 - 41
GLU	A+D	i_Rate_bsadj	25	0	0.0076	6.62	0 - 26
GLU	A+D	i_15min	25	0	0.008	6.57	0 - 29
GLU	A+D	expi_time	25	0	0.0082	6.77	0 - 40
GLU	A	iAUC_gAUC	25	0	0.0098	4.64	0 - 59
Milk	A	tot_pfy	25	3	0.0046	5.77	0 - 54
Milk	A+D	d120_cpct	25	20	0	11.54	7 - 33

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
Milk	A+D	d180_tppct	25	20	0.0001	11.11	6 - 34
Milk	A+D	tot_tppct	25	20	0.0002	11.18	4 - 33
Milk	A+D	d180_cppct	25	20	0.0003	12.14	7 - 33
Milk	A+D	d180_cpct	25	20	0.0003	11.41	6 - 34
Milk	A+D	d120_tppct	25	20	0.0005	10.23	3 - 37
Milk	A+D	tot_cppct	25	21	0	12.09	6 - 33
Milk	A+D	d60_cppct	25	21	0.0002	10.43	5 - 36
Milk	A+D	d120_cppct	25	21	0.0002	11.61	5 - 35
Milk	A+D	d270_cppct	25	21	0.0003	10.51	3 - 40
Milk	A+D	d240_tppct	25	21	0.0003	10.08	2 - 37
Milk	A+D	tot_cpct	25	21	0.0003	11.04	6 - 37
Milk	A+D	d60_cpct	25	21	0.0004	10.49	8 - 38
Milk	A+D	d240_cppct	25	21	0.0005	11.15	3 - 36
Milk	A+D	d270_tppct	25	21	0.0006	9.74	2 - 38
Milk	A+D	d240_cpct	25	21	0.0008	10.36	4 - 38
Milk	A+D	d60_tppct	25	21	0.0012	9.23	4 - 38
Milk	A	d180_cppct	25	22	0	10.61	7 - 33
Milk	A	d120_cppct	25	22	0.0001	10.11	5 - 35
Milk	A	d180_cpct	25	22	0.0001	10.18	6 - 34
Milk	A	d180_tppct	25	22	0.0002	9.53	6 - 34
Milk	A	d120_cpct	25	22	0.0002	10.19	7 - 33
Milk	A	d240_tppct	25	22	0.0006	8.76	2 - 37
Milk	A	d120_tppct	25	22	0.0007	8.73	3 - 37
Milk	A+D	d270_cpct	25	22	0.001	9.55	2 - 39
Milk	A	tot_cppct	25	23	0	10.8	6 - 33
Milk	A	tot_cpct	25	23	0.0001	10.08	6 - 37
Milk	A	d240_cppct	25	23	0.0002	9.78	3 - 36
Milk	A	d60_cpct	25	23	0.0002	9.36	8 - 38
Milk	A	d240_cpct	25	23	0.0002	9.32	4 - 38
Milk	A	tot_tppct	25	23	0.0003	9.83	4 - 33
Milk	A	d60_cppct	25	23	0.0004	9.26	5 - 36
Milk	A	d270_cppct	25	23	0.0005	9.25	3 - 40
Milk	A	d270_cpct	25	23	0.0005	8.62	2 - 39
Milk	A	d270_tppct	25	23	0.0006	8.4	2 - 38
Milk	A	d60_tppct	25	23	0.0008	7.99	4 - 38

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
GLU	A	G_time	25	29	0.0054	5.19	1 - 53
Milk	A	tot_pmy	25	41	0.0049	5.31	0 - 48
Milk	A	d60_pmy	25	42	0	9.81	0 - 48
Milk	A+D	d60_pmy	25	42	0.0001	11.6	0 - 48
Milk	A	d60_pfy	25	42	0.0001	11.61	0 - 52
Milk	A+D	d60_pfy	25	42	0.0001	12.82	0 - 52
Milk	A	tot_ply	25	42	0.0039	4.99	0 - 51
Milk	A+D	d60_pmsy	25	43	0.0002	11.46	0 - 51
Milk	A+D	tot_pmy	25	43	0.0015	8.08	0 - 48
Milk	A	d60_pmsy	25	44	0	9.51	0 - 51
Milk	A+D	d60_ply	25	44	0.0003	9.71	0 - 51
Milk	A	d60_ply	25	44	0.0004	8.13	0 - 51
Milk	A+D	tot_ply	25	44	0.001	8.12	0 - 51
Milk	A+D	tot_pmsy	25	44	0.004	6.98	0 - 53
Milk	A+D	tot_pfy	25	44	0.0047	7.05	0 - 54
Milk	A	d60_pwpy	25	46	0.0013	6.41	0 - 51
Milk	A+D	d60_pwpy	25	46	0.0021	7.44	0 - 51
Milk	A	d60_pcpy	25	46	0.0052	5.1	0 - 57
Milk	A+D	d60_ptpy	25	46	0.0054	6.62	0 - 56
Milk	A	d60_ptpy	25	46	0.0055	5.13	0 - 56
Milk	A+D	d60_pcpy	25	46	0.0065	6.54	0 - 57
Milk	A+D	tot_pwpy	25	46	0.0074	6.25	0 - 52
Milk	A	d60_pcy	25	46	0.0091	4.59	0 - 59
EEP	HS	GLY_RSQ	25	48	0.0043	3.9	0 - 58
GLU	HS	I_time	25	59	0.0097	3.65	0 - 59
TRH	HS	n_auc15_45	26	0	0.0039	3.93	0 - 28
TRH	HS	t_auc15_45	26	22	0.0002	5.36	7 - 59
TRH	HS	t_RATE	26	22	0.0011	4.14	7 - 56
TRH	HS	t_slope	26	23	0.0002	5.89	7 - 38
TRH	HS	t_auc_total	26	23	0.0025	4.23	4 - 59
TRH	HS	pk_GH_10	26	37	0.0008	4.28	0 - 48
TRH	HS	GHpk_mean	26	39	0.0024	3.87	0 - 59
GLU	HS	g_Totarea_60min	27	32	0.0098	3.34	6 - 49
EEP	A	glu_max	27	35	0.0029	5.22	6 - 39
GLU	HS	g_r_m_m5	27	35	0.0039	3.76	31 - 49

Table 9.6 Continued.

Trait	Analysis	Phenotype	Chr	Bst_lctn	Pval	Fval	95%CI
Milk	HS	d180_fpct	27	42	0.0009	4.49	0 - 49
Milk	HS	d270_fcpt	27	42	0.0016	4.27	0 - 49
Milk	HS	d240_fcpt	27	42	0.0017	4.32	0 - 49
TRH	HS	NEFA_bs	27	42	0.0025	3.89	17 - 49
Milk	HS	d120_mspct	27	42	0.0078	3.49	0 - 49
Milk	HS	d120_fpct	27	43	0.0003	4.84	1 - 49
Milk	HS	d60_mspct	27	43	0.0044	3.68	2 - 49
Milk	HS	d60_fpct	27	44	0.0002	4.88	3 - 49
EEP	A	glu_bs	27	45	0.0044	5.18	17 - 49
TRH	HS	t_RATE	27	48	0.0096	3.54	0 - 49
TRH	HS	t_auc15_45	27	49	0.0068	3.89	0 - 49
Milk	A	d120_fpct	27	49	0.0079	3.93	
Milk	A	d180_fpct	27	49	0.0084	3.87	
TRH	HS	t_slope	27	49	0.0092	3.79	0 - 49
EEP	HS	EPI_N_t_max	29	11	0.0069	3.63	1 - 68
TRH	A	t_emax_NEFA	29	13	0.0035	5.84	7 - 28

Table 9.8 Continued.

Symbol	Ensembl gene	Name/Description
PENK	ENSG00000181195	proenkephalin
PDE7A	ENSG00000205268	phosphodiesterase 7A
TOX		Thymus high mobility group box protein TOX
NSMAF	ENSG00000035681	Neutral sphingomyelinase (N-SMase) activation associated factor
FAM77D	ENSG00000185942	Family with sequence similarity 77, member D
MGC34646	ENSG00000177182	Hypothetical protein MGC34646; Cellular retinaldehyde-binding protein- like protein
	ENSG00000172825	No description
TMEM68	ENSG00000167904	transmembrane protein 68
	ENSG00000169122	No description
RDHE2	ENSG00000170786	epidermal retinal dehydrogenase 2
ARMC1	ENSG00000104442	armadillo repeat containing 1
IMPAD1	ENSG00000104331	myo-inositol monophosphatase A3, inositol monophosphatase domain containing 1
TGS1	ENSG00000137574	trimethylguanosine synthase homolog
MRPL15	ENSG00000137547	mitochondrial ribosomal protein L15
MOS	ENSG00000172680	Proto-oncogene serine/threonine-protein kinase mos (EC 2.7.1.37) (c- mos) (Oocyte maturation factor mos).
LYPLA1	ENSG00000120992	lysophospholipase I
LYN	ENSG00000147507	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog; Tyrosine-protein kinase Lyn
GGH	ENSG00000137563	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)
	ENSG00000189232	No description
	ENSG00000188561	No description
	ENSG00000188285	No description
	ENSG00000187863	No description
TCEA1	ENSG00000187735	No description
	ENSG00000186963	No description
	ENSG00000186904	No description
	ENSG00000184641	No description
	ENSG00000183226	No description
	ENSG00000183064	No description
	ENSG00000182699	No description
PSMC6P	ENSG00000181732	No description
	ENSG00000177203	No description

Table 9.8 Continued.

Symbol	Ensembl gene	Name/Description
	ENSG00000172823	No description
SEC11B	ENSG00000164738	SEC11 homolog B (S. cerevisiae)
DNAJC5B	ENSG00000147570	DnaJ (Hsp40) homolog, subfamily C, member 5 beta
CYP7B1	ENSG00000172817	cytochrome P450, family 7, subfamily B, polypeptide 1
CYP7A1	ENSG00000167910	cytochrome P450, family 7, subfamily A, polypeptide 1
CRH	ENSG00000147571	corticotropin releasing hormone
CHD7	ENSG00000171316	chromodomain helicase DNA binding protein 7
CHCHD7	ENSG00000170791	coiled-coil-helix-coiled-coil-helix domain containing 7
CA8	ENSG00000178538	carbonic anhydrase VIII
BHLHB5	ENSG00000180828	basic helix-loop-helix domain containing, class B, 5
ASPH		aspartate beta-hydroxylase
TRPA1	ENSG00000104321	transient receptor potential cation channel, subfamily A, member 1
TRAM1	ENSG00000067167	translocation associated membrane protein 1
TERF1	ENSG00000147601	Telomeric repeat binding factor 1, NIMA interacting protein 2
SULF1	ENSG00000137573	sulfatase 1, Extracellular sulfatase Sulf 1 precursor (EC 3.1.6.)
STAU2	ENSG00000040341	staufen, RNA binding protein, homolog 2 (Drosophila)
SLCO5A1	ENSG00000137571	solute carrier organic anion transporter family, member 5A1, Solute carrier family 21 member 15, Organic anion transporter polypeptide- related protein 4
SGK3	ENSG00000104205	serum/glucocorticoid regulated kinase family, member 3, Serine/threonine-protein kinase Sgk3
RPL7	ENSG00000147604	ribosomal protein L7
RDH10	ENSG00000121039	retinol dehydrogenase 10 (all-trans)
	ENSG00000182176	No description
RPESP	ENSG00000164764	RPE-spondin
	ENSG00000157556	No description
	ENSG00000178125	similar to RIKEN cDNA 1700011J18
TCF24	ENSG00000187728	transcription factor 24
PTTG3	ENSG00000178717	pituitary tumor-transforming 3
PRDM14	ENSG00000147596	PR domain containing 14
	ENSG00000178460	No description
	ENSG00000169085	No description
	ENSG00000165084	No description

Table 9.8 Continued.

Symbol	Ensembl gene	Name/Description
VCPIP1	ENSG00000175073	valosin containing protein (p97)/p47 complex interacting protein 1
CSPP1	ENSG00000104218	centrosome and spindle pole associated protein 1
	ENSG00000178778	No description
UBE2W	ENSG00000104343	ubiquitin-conjugating enzyme E2W (putative)
NCOA2	ENSG00000140396	nuclear receptor coactivator 2
MYBL1	ENSG00000185697	v-myb myeloblastosis viral oncogene homolog (avian)-like 1
MSC	ENSG00000178860	musculin (activated B-cell factor-1)
LACTB2	ENSG00000147592	lactamase, beta 2
KCNB2	ENSG00000182674	potassium voltage-gated channel, Shab-related subfamily, member 2
EYA1	ENSG00000104313	eyes absent homolog 1 (Drosophila)
H2AFZP2	ENSG00000187025	H2A histone family, member Z, pseudogene 2
	ENSG00000178144	No description
	ENSG00000176196	No description
	ENSG00000175953	No description
	ENSG00000168547	similar to ribosomal protein S15a
DEPDC2	ENSG00000046889	DEP domain containing 2
CPA6	ENSG00000165078	carboxypeptidase A6
COPS5	ENSG00000121022	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)
BTF3L2		basic transcription factor 3, like 2
ARFGEF1	ENSG00000066777	ADP-ribosylation factor guanine nucleotide-exchange factor 1(brefeldin A-inhibited)
ADHFE1	ENSG00000147576	alcohol dehydrogenase, iron containing, 1

Appendix H: Information on genes discarded from candidate gene study

LYPLA1 - lysophospholipase I

Function

Hydrolyzes fatty acids from S-acylated cysteine residues in proteins such as trimeric G alpha proteins or HRAS. Also has low lysophospholipase activity.

Catalytic activity: Palmitoyl-protein + H(2)O = palmitate + protein.

Gene Ontology (GO) terms:

Molecular function: catalytic activity; lysophospholipase activity; hydrolase activity

Biological process: lipid metabolism; fatty acid metabolism

Cellular component: membrane fraction; mitochondrion

Kegg Pathway for LYPLA1: hsa00564 Glycerophospholipid metabolism

Summary

Lysophospholipases are enzymes that act on biological membranes to regulate the multifunctional lysophospholipids. The protein encoded by this gene hydrolyzes lysophosphatidylcholine in both monomeric and micellar forms. The use of alternate polyadenylation sites has been found for this gene. There are alternatively spliced transcript variants described for this gene but the full length nature is not known yet.

Potassium voltage-gated channel, Shab-related subfamily, member 2 (KCNB2)

KCNB2 is a delayed rectifier, voltage-gated potassium (Kv) channel which is a multi-pass membrane protein that mediates the voltage-dependent potassium ion permeability of excitable membranes. Voltage-gated potassium channels open in response to membrane depolarisation to allow rapid diffusion of K+ ions out of the cell, thus repolarising the cell to restore a negative resting membrane potential. OR Channels open or close in response to the voltage difference across the membrane, letting potassium ions pass in accordance with their electrochemical gradient

Voltage-gated potassium (Kv) channels diverse functions include regulating neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction, and cell volume. Four sequence-related potassium channel genes - shaker, shaw, shab, and shal - have been identified in Drosophila, and each has been shown to have human homolog(s). This gene encodes a member of the potassium channel, voltage-gated, shab-related subfamily. This member is a delayed rectifier potassium channel. The gene is expressed in gastrointestinal smooth muscle cells.

Use of N-terminal anti-body staining of rat pancreatic islet cells revealed that Kv2.2 was present in somatostatin-containing δ cells and glucagon-containing α cells (Wolf-Goldberg *et al.* 2006). Thus may play some type of regulatory role within these cells in relation to nutrient partitioning.

γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter

Recent data suggest that GABA neurotransmission can be excitatory in basal conditions, not only in immature but also in adult tissue. GABAergic synapses are indeed endowed with a unique and fundamental feature – resulting from their ClK permeability – that enables them to shift from an inhibitory mode of operation to one that mainly excites.

CPA6 – carboxypeptidase A6

Function

Subcellular location: Secreted protein (By similarity)

Gene Ontology (GO) terms:

Molecular function: carboxypeptidase activity; carboxypeptidase A activity; metallopeptidase activity; zinc ion binding; metal ion binding

Biological process: proteolysis

Catalysis of the reaction: peptidyl-L-amino acid + H2O = peptide + L-amino acid. Little or no action with -Asp, -Glu, -Arg, -Lys or -Pro

Summary

Carboxypeptidases have functions ranging from digestion of food to selective biosynthesis of neuroendocrine peptides. Members of the A/B subfamily of carboxypeptidases, such as CPA6,

contain an approximately 90-amino acid pro region that assists in the folding of the active carboxypeptidase domain. Cleavage of the pro region activates the enzyme (Wei et al., 2002).

SULF1 – sulfatase 1

Subcellular location: endoplasmic reticulum, Golgi stacks, plasma membrane

Protein Family, Domain: Arylsulfatase, enzyme, N-acetylglucosamine-6-sulfatase, Sulfuric ester hydrolase

Regulates: Erk, MET, AKT

Regulated by: SUMF1, 1-alpha, 25-dihydroxy vitamin D3

Role in cell: invasion, apoptosis, mitogenecity, contact growth inhibition, motility

Function

Exhibits arylsulfatase activity and highly specific endoglucosamine-6-sulfatase activity. It can remove sulfate from the C-6 position of glucosamine within specific subregions of intact heparin. Diminishes heparan sulfate proteoglycans (HSPG) sulfation, inhibits signalling by heparin-dependent growth factors, diminishes proliferation, and facilitates apoptosis in response to exogenous stimulation

Summary

HSPGs act as coreceptors for numerous heparin-binding growth factors and cytokines and are involved in cell signalling. Heparan sulfate 6-O-endosulfatases, such as SULF1, selectively remove 6-O-sulfate groups from heparin sulfate. This activity modulates the effects of heparan sulfate by altering binding sites for signalling molecules (Dai et al., 2005).

Apoptosis is likely to be more important after the first 60 days of lactation.

TGS1 – trimethylguanosine synthase homolog (S. cerevisiae)

role in transcriptional regulation

Function

May be a methyltransferase. Binds S-adenosyl-L- methionine and RNA. Plays a role in transcriptional regulation

Gene Ontology (GO) terms:

Molecular function: molecular function unknown; methyltransferase activity; S-adenosylmethionine-dependent methyltransferase activity; transferase activity

Cellular component: nucleus

Biochem Biophys Res Commun. 2003 Sep 12;309(1):44-51. Links

Different isoforms of PRIP-interacting protein with methyltransferase domain/trimethylguanosine synthase localizes to the cytoplasm and nucleus.

Enünlü I, Pápai G, Cserpán I, Udvardy A, Jeang KT, Boros I.

A protein family including the recently identified PIMT/Tgs1 (PRIP-interacting protein with methyltransferase domain/trimethylguanosine synthase) was identified by searching databases for homologues of a newly identified Drosophila protein with RNA-binding activity and methyltransferase domain. Antibodies raised against a short peptide of the mammalian homologue show a 90-kDa isoform expressed specifically in rat brain and testis and a 55-kDa form expressed ubiquitously. In HeLa cells, the larger isoform of the protein is nuclear and associated with a 600-kDa complex, while the smaller isoform is mainly cytoplasmic and co-localizes to the tubulin network. Inhibition of PIMT/Tgs1 expression by siRNA in HeLa cells resulted in an increase in the percentage of cells in G2/M phases. In yeast two-hybrid and *in vitro* GST pull down experiments, the conserved C-terminal region of PIMT/Tgs1 interacted with the WD domain containing EED/WAIT-1 that acts as a polycomb-type repressor in the nucleus and also binds to integrins in the cytoplasm. Our experiments, together with earlier data, indicate that isoforms of the PIMT/Tgs1 protein with an RNA methyltransferase domain function both in the nucleus and in the cytoplasm and associate with both elements of the cytoskeletal network and nuclear factors known to be involved in gene regulation.

MYBL1 – v-myb myeloblastosis viral oncogene homolog (avian)-like 1

Regulation of transcription, transcription factor

Protein Family, Domain: core domain, functional, SANT domain, transcription activation domain, transcription regulator

Subcellular Location: nuclear fraction, Nucleus

Regulates: MYC, BCL2, TFEC, EMILIN2, TIMM44, ACTN1, PSD4, HSPA8, CD53, CASP6, SET, API5, IQGAP1 (includes EG:8826), SEC31L1, BET1L

Regulated by: beta-estradiol, amphetamine, SOD1, EGR1, Tax, raloxifene, RELA, NFKB1, TP53, EGR4, trans-hydroxytamoxifen, NLK

Binds: Nfy, BCL2, SP1, NLK, CREBBP

Role in cell: apoptosis, growth, expansion, S phase

Disease: hyperplasia, hypoplasia, neoplasia, cancer, gastric cancer, gastric carcinoma

Function

Strong transcriptional activator; DNA-binding protein that specifically recognise the sequence 5'-YAAC[GT]G-3'. Could have a role in the proliferation and/or differentiation of neurogenic, spermatogenic and B-lymphoid cells.

Genatlas biochemistry entry for MYBL1: avian myeloblastosis viral (v-myb) oncogene homolog-like 1

Gene Ontology (GO) terms:

DNA binding; nucleus; regulation of transcription; transcriptional activator activity

Oncogene. 1994 Sep;9(9):2469-79.Links

The human A-myb protein is a strong activator of transcription.

Golay J, Loffarelli L, Luppi M, Castellano M, Introna M.

The A-myb gene belongs to the family of the c-myb proto-oncogene. We report here the cloning from a B lymphocyte cDNA library of the previously missing 3' half of the human A-myb cDNA, thus closing the previously still incomplete open reading frame. Analysis of the homologies between the different myb proteins reveals four domains of high conservation. We show, using a polyclonal rabbit antibody, that the 90 kd human A-myb protein is nuclear and that it activates transcription from the KHK-CAT reporter 6-10 times more strongly than c-myb in NIH3T3 cells. The transactivating function of A-myb depends on the presence of the myb

binding site in the reporter, and on both the DNA binding and acidic domains of the A-myb protein. The bacterially expressed protein protects the myb binding sites of the reporter in footprint experiments. Binding of the A-myb protein is shown in gel retardation assays to be specific for the classical c-myb recognition sequence PyAACG/TG. In addition, like c-myb, A-myb binds more strongly to the MIM-A synthetic oligonucleotide that carries the TAACGG sequence than to the MBS-I oligonucleotide containing TAAGTG. Finally, DNA binding activity is demonstrated to require the N-terminal portion of the protein containing the three tandem repeats of amino acids conserved in all myb proteins. We have thus shown that the A-myb protein is a strong activator of transcription and that this activity depends on both the DNA-binding and acidic domains.

Appendix I: Phenotypic correlations

Table 9.9 Pearson Correlation Coefficients between glucose challenge response (across the top, explanation of abbreviation in Table 9.4) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	i_base	i_AUC _45min	i_AUC 15min	i_10min	i_15min	i_max	i_time	ii_time
dim	0.108							
	0.0046							
	682							
tot_pfy		0.081	0.084	0.078		0.082		0.081
		0.0388	0.0313	0.0417		0.0311		0.0345
		650	660	677		686		684
d240_pfy			0.079	0.076		0.07802		
			0.0421	0.0475		0.0411		
			660	677		686		
d270_pfy		0.078	0.079	0.076		0.0752		
		0.0464	0.0429	0.0478		0.049		
		650	660	677		686		
d300_pfy		0.078						
		0.0457						
		650						
tot_pcpy	0.109	0.088	0.088	0.083				
	0.0043	0.025	0.0235	0.0318				
	682	650	660	677				
d180_pcpy	0.098		0.077					
	0.104		0.0486					
	682		660					
d240_pcpy	0.092	0.086	0.087	0.084	0.081		-0.07535	
	0.0167	0.029	0.0248	0.028	0.0353		0.0489	
	682	650	660	677	676		684	
d270_pcpy	0.078	0.089	0.088	0.085	0.081		-0.07668	
	0.0426	0.023	0.024	0.0275	0.0342		0.045	
	682	650	660	677	676		684	
d300_pcpy		0.089	0.085	0.082	0.079		-0.07521	
		0.0232	0.0292	0.034	0.04		0.0493	
		650	660	677	676		684	
tot_ptpy	0.108	0.087	0.087	0.082				
	0.0046	0.0273	0.0248	0.0334				
	682	650	660	677				
d180_ptpy	0.097							
	0.0114							
	682							
d240_ptpy	0.091	0.084	0.086	0.083	0.080			
	0.018	0.0325	0.0269	0.0302	0.0384			
	682	650	660	677	676			

Table 9.10 Pearson Correlation Coefficients between glucose challenge response (across the top, explanation of abbreviation in Table 9.4) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	i_base	i_AUC_45min	i_AUC 15min	i_10min	i_15min	i_max	i_time	ii_time
d270_ptpy	0.077 0.0452 682	0.087 0.0265 650	0.086 0.0265 660	0.083 0.0302 677	0.080 0.0382 676		-0.07565 0.048 684	
d300_ptpy		0.087 0.0271 650	0.083 0.0323 660	0.080 0.0375 677	0.077 0.0453 676			
tot_pcy	0.109 0.0043 682	0.092 0.0185 650	0.092 0.0182 660	0.086 0.0249 677	0.078 0.0419 676	0.075 0.0482 686		0.075 0.0489 684
d120_pcy	0.077 0.0434 682							
d180_pcy	0.100 0.0089 682	0.078 0.0474 650	0.082 0.0355 660	0.079 0.0388 677	0.078 0.042 676			
d240_pcy			0.091 0.0194 660	0.088 0.0221 677	0.085 0.0279 676			
d270_pcy	0.077 0.0435 682	0.093 0.0178 650	0.091 0.0196 660	0.088 0.0225 677	0.084 0.0281 676		-0.080 0.0358 684	
d300_pcy		0.092 0.0189 650	0.087 0.0248 660	0.084 0.0287 677	0.082 0.034 676		-0.078 0.0404 684	
tot_pwpy	0.084 0.0285 682							
tot_ply	0.103 0.0069 682							
d180_ply	0.093 0.0146 682							
d240_ply	0.091 0.0177 682							
d270_ply	0.081 0.0348 682							

Table 9.11 Pearson Correlation Coefficients between glucose challenge response (across the top, explanation of abbreviation in Table 9.4) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	g_base	i_base	i_AUC 15min	i_10min	i_t_max	i_time	ii_time	E_H	N_t_min
d60_fpct	0.078 0.0416 682								
tot_cppct							0.080 0.0366 684		
d60_cppct								-0.092 0.0184 656	
d120_cppct								-0.079 0.0441 656	
tot_tppct							0.075 0.0485 684		
d60_tppct								-0.087 0.0251 656	
d120_tppct								-0.077 0.0491 656	
tot_cpct			0.078 0.0453 660	0.083 0.03 677		0.076 0.0469 684	0.087 0.0223 684		
d240_cpct							0.077 0.0433 684		
d270_cpct							0.077 0.0453 684		
tot_wppct					0.090 0.0182 686				
d60_wppct					0.079 0.039 686			-0.102 0.0092 656	
d120_wppct								-0.098 0.0117 656	

Table 9.12 Pearson Correlation Coefficients between glucose challenge response (across the top, explanation of abbreviation in Table 9.4) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	g_base	i_base	i_AUC 15min	i_10min	i_t_max	i_time	ii_time	E_H	N_t_min
d180_wppct					0.087 0.0224 686			-0.084 0.0324 656	
d240_wppct					0.089 0.0204 686			-0.077 0.0484 656	
d270_wppct					0.091 0.0172 686			-0.078 0.0445 656	0.076 0.0468 686
tot_lpct		0.098 0.0105 682							
d60_lpct		0.087 0.0235 682							
d120_lpct		0.104 0.0067 682							
d180_lpct		0.108 0.0047 682							
d240_lpct		0.111 0.0037 682							
d270_lpct		0.115 0.0026 682							

Table 9.13 Pearson Correlation Coefficients between adrenaline challenge response (across the top, explanation of abbreviation in Table 9.3) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	GLY_t_max	GLY_base	N_max	N_pk_15
dim				0.081
				0.0357
				677
tot_pfy	-0.098		0.091	0.111
	0.0106		0.018	0.0038
	681		681	677
d60_pfy	-0.107	0.081		
	0.0053	0.0341		
	681	681		
d120_pfy	-0.106	0.0837		0.08024
	0.0056	0.0289		0.0369
	681	681		677
d180_pfy	-0.110	0.087	0.086	0.093
	0.0039	0.0233	0.0256	0.0158
	681	681	681	677
d240_pfy	-0.099	0.083	0.080	0.089
	0.0096	0.0303	0.0359	0.02
	681	681	681	677
d270_pfy	-0.088	0.078		0.081
	0.0211	0.0421		0.0341
	681	681		677
d300_pfy	-0.077			
	0.0434			
	681			
d60_pcpy	-0.081			
	0.035			
	681			
d120_pcpy	-0.079			
	0.0404			
	681			
d180_pcpy	-0.083			
	0.0308			
	681			
d60_ptpy	-0.081			
	0.0351			
	681			
d120_ptpy	-0.076			
	0.0473			
	681			

Table 9.14 Pearson Correlation Coefficients between adrenaline challenge response (across the top, explanation of abbreviation in Table 9.3) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	GLY_t_max	GLY_base	N_max	N_pk_15
d180_ptpy	-0.080			
	0.0358			
	681			
d60_pcy	-0.079			
	0.0387			
	681			
d120_pcy	-0.075			
	0.0497			
	681			
d180_pcy	-0.078			
	0.0418			
	681			
tot_pwpy	-0.076			
	0.0467			
	681			
d180_pwpy	-0.079			
	0.0393			
	681			
d60_ply	-0.077			
	0.0457			
	681			

Table 9.15 Pearson Correlation Coefficients between adrenaline challenge response (across the top, explanation of abbreviation in Table 9.3) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	N_bs	N_pk_15	N_auc	GLY_auc 15_60	N_t_max
d60_fpct	0.099 0.0095 681	0.108 0.0048 677			
d120_ fpct	0.106 0.0058 681	0.101 0.0086 677			
d180_ fpct	0.107 0.0051 681	0.088 0.0221 677			
d240_ fpct	0.106 0.0058 681				
d270_ fpct	0.105 0.0063 681				
d180_cppct			-0.079 0.045 644		
d240_cppct			-0.085 0.0305 644		
d270_cppct			-0.088 0.026 644		

Table 9.16 Pearson Correlation Coefficients between adrenaline challenge response (across the top, explanation of abbreviation in Table 9.3) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	N_bs	N_pk_15	N_auc	GLY_auc 15_60	N_t_max
d180_tppct			-0.079 0.0461 644		
d240_tppct			-0.084 0.0329 644		
d270_tppct			-0.086 0.0286 644		
tot_cpct			-0.080 0.0423 644		
d120_cpct			-0.083 0.0355 644		
d180_cpct			-0.088 0.0255 644		
d240_cpct			-0.093 0.0182 644		
d270_cpct			-0.097 0.0134 644		
d180_wppct					0.076 0.0489 681
d60_lpct				-0.078 0.0465 657	
d120_lpct				-0.077 0.0493 657	

Table 9.17 Pearson Correlation Coefficients between TRH challenge response (across the top, explanation of abbreviation in Table 9.5) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	t_ AUC	t_auc15	t_auc 15_45	t_pk_ mean	t_RATE	t_slope	NEFA_ base	NEFA _max	n_pk_ mean_ bsadj
tot_pfy	-0.091 0.0176 680	-0.096 0.0119 686		-0.079 0.038 690			0.122 0.0013 690		-0.077 0.046 678
d60_pfy	-0.136 0.0004 680	-0.120 0.0017 686	-0.125 0.001 683	-0.133 0.0004 690	-0.099 0.009 690	0.116 0.0022 693	0.082 0.0312 690		
d120_pfy	-0.130 0.0007 680	-0.118 0.0019 686	-0.114 0.0028 683	-0.123 0.0012 690	-0.090 0.0175 690	0.104 0.0059 693	0.095 0.0124 690		
d180_pfy	-0.126 0.001 680	-0.119 0.0018 686	-0.106 0.0058 683	-0.114 0.0027 690	-0.083 0.0289 690	0.094 0.013 693	0.114 0.0028 690		
d240_pfy	-0.115 0.0026 680	-0.111 0.0037 686	-0.094 0.0137 683	-0.102 0.0071 690		0.081 0.0331 693	0.121 0.0014 690	0.077 0.0428 693	
d270_pfy	-0.106 0.0055 680	-0.103 0.007 686	-0.088 0.0222 683	-0.095 0.0122 690			0.118 0.0018 690	0.080 0.0346 693	
d300_pfy	-0.098 0.011 680	-0.094 0.0133 686	-0.081 0.0345 683	-0.088 0.0203 690			0.113 0.0029 690	0.081 0.0326 693	
tot_pcpy	-0.082 0.0324 680						0.0898 0.0183 690	0.033 0.383 693	
d60_pcpy	-0.116 0.0025 680		-0.102 0.0075 683	-0.093 0.0144 690		0.07879 0.0381 693			
d120_pcpy	-0.114 0.0029 680		-0.097 0.0113 683	-0.090 0.0177 690					
d180_pcpy	-0.112 0.0033 680	-0.075 0.0498 686	-0.091 0.0172 683	-0.085 0.0252 690			0.08255 0.0301 690		
d240_pcpy	-0.104 0.0065 680		-0.083 0.0304 683	-0.079 0.0392 690			0.08817 0.0205 690		
d270_pcpy	-0.098 0.0107 680		-0.078 0.0412 683	-0.075 0.0492 690			0.08696 0.0223 690		

Table 9.18 Pearson Correlation Coefficients between TRH challenge response (across the top, explanation of abbreviation in Table 9.5) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	t_ AUC	t_auc15	t_auc 15_45	t_pk_ mean	t_RATE	t_slope	NEFA_ base	NEFA _max	n_pk_ mean_ bsadj
d300_pcpy	-0.091						0.08427		
	0.0176						0.0269		
	680						690		
tot_ptpy	-0.081						0.08866		
	0.0346						0.0198		
	680						690		
d60_ptpy	-0.113		-0.101	-0.092		0.077			
	0.0031		0.0085	0.016		0.0429			
	680		683	690		639			
d120_ptpy	-0.112		-0.096	-0.090					
	0.0034		0.012	0.0184					
	680		683	690					
d180_ptpy	-0.112		-0.091	-0.085			0.0809		
	0.0036		0.0174	0.0251			0.0336		
	680		683	690			690		
d240_ptpy	-0.103		-0.082	-0.078			0.08652		
	0.0072		0.0314	0.0393			0.023		
	680		683	690			690		
d270_ptpy	-0.096		-0.077	-0.075			0.08548		
	0.0118		0.0431	0.0498			0.0247		
	680		683	690			690		
d300_ptpy	-0.090						0.08326		
	0.0193						0.0288		
	680						690		

Table 9.19 Pearson Correlation Coefficients between TRH challenge response (across the top, explanation of abbreviation in Table 9.5) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	t_AUC	t_auc15	t_auc 15_45	t_pk_ mean	t_slope	NEFA_ base	n_auc15	n_slope
tot_pcy	-0.078 0.0432 680					0.089 0.0192 690		
d60_ pcy	-0.109 0.0046 680		-0.097 0.0111 683	-0.089 0.0199 690	0.076 0.0464 693			
d120_ pcy	-0.107 0.0052 680		-0.092 0.0166 683	-0.086 0.0236 690				
d180_ pcy	-0.105 0.006 680		-0.086 0.0246 683	-0.082 0.032 690		0.082 0.0319 690		
d240_ pcy	-0.098 0.0105 680		-0.079 0.0398 683	-0.076 0.0457 690		0.087 0.0222 690		
d270_ pcy	-0.093 0.0156 680					0.086 0.0238 690		
d300_ pcy	-0.08697 0.0233 680					0.084 0.0279 690		
tot_pwpy	-0.098 0.0106 680	-0.079 0.0376 686	-0.076 0.0459 683			0.082 0.0308 690		0.085 0.0248 693
d60_ pwpy	-0.118 0.0021 680	-0.080 0.037 686	-0.103 0.0072 683	-0.094 0.0135 690				
d120_ pwpy	-0.120 0.0017 680	-0.082 0.0321 686	-0.104 0.0067 683	-0.095 0.0122 690				
d180_ pwpy	-0.127 0.0009 680	-0.090 0.0189 686	-0.105 0.0061 683	-0.095 0.0126 690				

Table 9.20 Pearson Correlation Coefficients between TRH challenge response (across the top, explanation of abbreviation in Table 9.5) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	t_AUC	t_auc15	t_auc 15_45	t_pk_ mean	t_slope	NEFA_ base	n_auc15	n_slope
d240_ pwpy	-0.118	-0.085	-0.095	-0.087		0.075		0.075
	0.0021	0.026	0.0133	0.0227		0.0491		0.0476
	680	686	683	690		690		693
d270_ pwpy	-0.109	-0.080	-0.087	-0.081				0.075
	0.0044	0.0371	0.0222	0.033				0.0487
	680	686	683	690				693
d300_ pwpy	-0.101	-0.075	-0.081	-0.076				
	0.0084	0.0497	0.0352	0.047				
	680	686	683	690				
tot_ply	-0.085	-0.078				0.078		
	0.0273	0.0415				0.0395		
	680	686				690		
d60_ply	-0.131	-0.089	-0.112	-0.097	0.075		-0.078	
	0.0006	0.0191	0.0034	0.0112	0.0475		0.0433	
	680	686	683	690	693		664	
d120_ply	-0.121	-0.09	-0.096	-0.087			-0.079	
	0.0016	0.0184	0.0119	0.023			0.0407	
	680	686	683	690			664	
d180_ply	-0.113	-0.090	-0.084	-0.077		0.077	-0.079	
	0.0033	0.0185	0.0285	0.0444		0.0441	0.0409	
	680	686	683	690		690	664	
d240_ply	-0.102	-0.086				0.079		
	0.008	0.024				0.0382		
	680	686				690		
d270_ply	-0.095	-0.083				0.077		
	0.0128	0.0304				0.0436		
	680	686				690		
d300_ply	-0.089	-0.078						
	0.0201	0.04						
	680	686						

Table 9.21 Pearson Correlation Coefficients between TRH challenge response (across the top, explanation of abbreviation in Table 9.5) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	prlc_t_max	prlc_max	NEFA_max	pk_NEFA_10	GH_RATE	GH_slope	GH_auc 15_30
d60_fpct	0.078			0.082			
	0.0403			0.0319			
	693			689			
d120_fpct	0.083		0.075	0.090			
	0.0294		0.0476	0.018			
	693		693	689			
d180_fpct	0.084		0.080	0.096			
	0.0266		0.036	0.0116			
	693		693	689			
d240_fpct	0.090		0.087	0.100			
	0.0174		0.0226	0.0083			
	693		693	689			
d270_fpct	0.092		0.089	0.102			
	0.0159		0.0188	0.0077			
	693		693	689			
tot_cppct		0.075				0.076	
		0.0485				0.0466	
		693				693	
d240_cppct						0.079	
						0.0368	
						693	
d270_cppct					-0.078	0.084	
					0.0419	0.0267	
					687	693	
d240_tppct						0.079	
						0.0375	
						693	
d270_tppct					-0.078	0.083	
					0.0411	0.0296	
					687	693	
d240_cpct						0.079	
						0.0381	
						693	
d270_cpct						0.082	-0.075
						0.0304	0.0489
						693	683

Table 9.22 Pearson Correlation Coefficients between TRH challenge response (across the top, explanation of abbreviation in Table 9.5) and milk production phenotypes (down the left side, explanation of abbreviations in Table 9.7), followed by the probability that R is different from zero and the number of observations.

	prlc_t_max	GH_RATE	p_AUC	p_AUC15_30	p_slope	IGF-I base
tot_wppct		-0.075 0.049 687				
d60_wppct	0.089 0.0191 693	-0.090 0.0182 687				
d120_wppct	0.082 0.0307 693	-0.079 0.0391 687				
d180_wppct		-0.078 0.0414 687				
d240_wppct		-0.077 0.0385 687				
d270_wppct		-0.077 0.0423 687				
tot_lpct			-0.085 0.027 682	-0.093 0.0147 688	0.080 0.0341 693	-0.101 0.0076 691
d60_lpct				-0.078 0.0421 688	0.078 0.0396 693	-0.075 0.0482 691
d120_lpct				-0.079 0.0386 688	0.077 0.0431 693	-0.088 0.0208 691
d180_lpct				-0.082 0.032 688	0.077 0.0417 693	-0.093 0.0148 691
d240_lpct			-0.079 0.0393 682	-0.089 0.0199 688	0.080 0.0343 693	-0.097 0.0111 691
d270_lpct			-0.082 0.0328 682	-0.091 0.0168 688	0.080 0.035 693	-0.096 0.0117 691