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The Role of Insulin in the Regulation of Milk Protein Synthesis in Pasture-fed Lactating Ruminants

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Abstract

The primary aim of this thesis was to determine the role of insulin in milk protein production in pasture-fed lactating ruminants (ewes and cows), using the hyperinsulinaemic euglycaemic clamp (HEC) technique.

Three experiments were carried out. In the first 2 experiments, the response of pasture-fed ewes and dairy cows to the HEC were established and compared to concentrate-fed ruminants (dairy cows and goats). Use of the HEC technique in pasture-fed ruminants did not result in an increase in milk protein yield or concentration. However, a reduction in feed intake along with maintenance of milk protein yield resulted in a change in efficiency of utilisation of dietary crude protein for milk protein production. This indicated that changes in blood insulin could result in changes in nutrient partitioning to maintain milk protein production.

In Experiment 3, mechanisms were examined that could maintain milk protein production despite a reduction in feed intake. The arterio-venous concentration difference technique and a leucine tracer infusion were used to measure amino acid (AA) uptake and subsequent metabolism for milk protein production under conditions imposed by the HEC. This experiment demonstrated that the HEC reduced AA supply to the mammary gland and there was a decrease in the uptake of some AA. There was no increase in mammary blood flow to compensate for this. The deficit in the ratio of AA uptake to their secretion in milk protein suggests the use of plasma free AA concentrations underestimates uptake of AA by the mammary gland and there are contributions by alternative sources such as peptide AA and erythrocytes. There was no decrease in leucine oxidation in the mammary gland, indicating that AA were not conserved for milk protein production through an alteration in this mechanism. These results support the theory that the mammary gland has the ability to respond to modified precursor supply to maintain milk protein output.

Thesis summary

The primary aim of this thesis was to determine the role of insulin in milk protein production in pasture-fed lactating ruminants (ewes and cows). To do this, the hyperinsulinaemic euglycaemic clamp (HEC) technique was utilised. This technique uses simultaneous infusions of insulin and glucose so that the role of insulin can be examined without the confounding effects of hypoglycaemia.

In the first two experiments, abomasally-cannulated ewes and rumen fistulated Jersey cows were subjected to a HEC with or without an abomasal infusion of supplemental protein (in the form of casein) in a two period cross-over design experiment.

In the experiment with lactating ewes (Chapter 3), the casein infusion resulted in significantly higher milk and milk protein yield. However, there was no increase in milk or milk protein yield with the subsequent HEC. Feed intake was significantly depressed during the HEC but as milk protein output was maintained, this resulted in an increase in the efficiency of dietary protein used for milk protein synthesis. The HEC caused a decrease in circulating concentrations of essential amino acids (EAA), particularly the branched chain AA (BCAA), leucine, isoleucine and valine.

In the experiment with lactating dairy cows (Chapter 4), there was no increase in milk or milk protein yield in response to the casein infusion. Furthermore, there was no milk protein response to the HEC in the casein-supplemented cows. However, the HEC caused milk and milk protein yield to decrease in the non-supplemented cows. As in the study with lactating ewes, feed intake was significantly reduced by the HEC, which resulted in an increase in the efficiency of dietary crude protein used for milk protein production. The HEC also reduced circulating EAA concentrations in the cows.

The data generated in both these experiments showed similar changes in variables such as changes in circulating concentrations of amino acids (AA) and energy metabolites to those observed in concentrate-fed animals where a milk protein response to the HEC alone or HEC plus supplemental protein was demonstrated. It was not clear why there was no such response in the pasture-fed animals but it may have been due to species

differences, a stage of lactation effect or the pasture-fed animals being in negative energy balance.

The third experiment used the arterio-venous (A-V) concentration difference technique and a tracer infusion of ^{13}C-leucine to examine AA uptake and subsequent metabolism for milk protein production under HEC conditions in lactating ewes. It was hypothesised that insulin (by use of the HEC without supplemental protein) stimulated an increase in AA uptake by the mammary gland, increased AA supply to the gland by increasing blood flow, and decreased AA oxidation within the gland so that AA were conserved for use in milk protein production.

As with the first two experiments, there was no increase in milk or milk protein yield under HEC conditions. The arterial supply of AA to the mammary gland was reduced but there was no change in mammary blood flow to compensate for this. Actual uptake of some EAA was reduced in insulin treated ewes. The deficit in the ratio of AA uptake to their secretion in milk protein suggests the use of plasma free AA concentrations underestimates uptake of AA by the mammary gland and there are contributions by alternative sources such as peptide AA and erthrocytes. There was no decrease in leucine oxidation in the mammary gland, indicating that AA were not conserved for milk protein production through an alteration in this mechanism. The leucine kinetics showed a tendency ($P=0.08$) for difference in irreversible loss rate but not the partitioning of leucine to the mammary gland between the control and HEC ewes. In the mammary gland, there was a lower uptake of leucine in the HEC treated ewes but no change in leucine oxidation. Although the HEC decreased total protein synthesis in the mammary gland, the ratio of leucine secreted in milk protein:gland protein synthesis was similar between the insulin treated (0.65) and control ewes (0.71), suggesting that insulin did not alter the transfer of leucine into milk protein.

These results support the theory that the mammary gland has the ability to respond to modified precursor supply to maintain milk protein output. These results are discussed in relation to work done with concentrate-fed animals.

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AA	Amino acid
ANOVA	Analysis of variance
BCAA	Branch chain amino acids
BF	Blood flow
BW	Body weight
C_A	Arterial concentration of $C0_2$
CO_2	Carbon dioxide
CP	Crude protein
C_V	Venous concentration of $C0_2$
CV	Coefficient of variation
d	Day
DM	Dry matter
DMI	Dry matter intake
EAA	Essential amino acid
$E_{C,A}$	Isotopic enrichment of arterial CO_2
$E_{C,V}$	Isotopic enrichment of venous CO_2
$E_{K,A}$	Isotopic enrichment of arterial KIC
$E_{K,V}$	Isotopic enrichment of venous KIC
$E_{L,A}$	Isotopic enrichment of arterial leucine
$E_{L,V}$	Isotopic enrichment of venous leucine
Ey	Isotopic enrichment of either venous leucine or KIC as the precursor pool
g	Gram
H_2	Hydrogen
Ha	Hectare
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
He	Helium
HEC	Hyperinsulinaemic euglycaemic clamp
HNO_3	Nitric acid
HPLC	High performance liquid chromatography

h	Hour
ICP	Inductive coupled plasma emission spectometry
IE	Isotopic enrichment
IGF-1	Insulin-like growth factor-1
ILR	Irreversible loss rate
ILR_{ALEU}	Irreversible loss rate of arterial leucine
K_A	Arterial concentration of KIC
kg	Kilogram
KIC	Keto isocaproate acid
KOH	Potassium hydroxide
K_V	Venous concentration of KIC
L_A	Arterial concentration of leucine
Leu	Leucine
LO	Leucine oxidation
LSMeans	Least squares means
L_V	Venous concentration of leucine
MBF	Mammary blood flow
mg	Milligram
min	Minute
MJ ME	Mega joules metabolisable energy
ml	Millilitre
mm	Millimetre
mM	Millimole
MPE	Moles percent excess
MPE_{ALEU}	Mole percent excess of arterial leucine
MS	mass spectometer
N	Nitrogen
Na_2EDTA	Disodium ethylenediaminetetraacetate
NEAA	Non essential amino acids
NEFA	Non esterfied fatty acids
ng	Nanogram
NIRS	Near infrared reflectance spectroscopy
NITS	Near infrared transmittance spectroscopy
NO_2	Nitrous oxide

NPN	Non protein nitrogen
NRC	National research council
O_2	Oxygen
P	Probability
pg	Picogram
RIA	Radioimmunoassay
RPM	Revolutions per minute
SCC	Somatic cell count
SD	Standard deviation
SEM	Standard error of the mean
SSA	Sulphosalicylic acid
Sy	Precursor pool for leucine or KIC
t	Time
TCA	Trichloroacetic acid
µl	Microlitre
UV	Ultra violet
VFA	Volatile fatty acid

Chapter 1 Introduction.

1.1 Background

The current drive in the dairy industry worldwide is to increase milk protein production as this milk component is increasing in value relative to milk fat. This is seen in the advanced payout announced by Fonterra in New Zealand of 229.16 cents/kg and 545.62 cents/kg for fat and protein respectively for the 2001/2002 season (Fonterra website, www.fonterra.com, March 2002). It is also driven by the consumers' general perception of protein as being healthier than fat. The challenge that the dairy industry faces is to consistently increase milk protein production, either through an increase in milk protein yield or concentration. However, our current understanding of dietary utilisation for milk production does not allow reliable management of dairy cows to consistently increase milk protein production above that currently achieved with standard rations.

Many studies have attempted to increase milk protein synthesis empirically through increasing the supply of precursors (amino acids) to the mammary gland (Sutton 1989), but few have examined the mechanisms that regulate milk protein synthesis with the objective of devising more effective strategies of increasing milk protein yield. McGuire *et al.* (1995) demonstrated that manipulating insulin status by use of the hyperinsulinaemic euglycaemic clamp (HEC) technique in concentrate-fed cows increased milk protein yield. Subsequent studies have shown that when insulin status was manipulated in conjunction with supplementary dietary protein, milk protein yield was substantially increased (25-28%, Griinari *et al.* 1997, Mackle *et al.* 1999, 2000a, Bequette *et al.* 2001). These results are remarkable when considered relative to the adaptations of whole body metabolism to meet the requirements for lactation, which primarily seem to be aimed at blunting the effects of insulin, particularly in adipose and muscle tissue. It still needs to be established if insulin regulates pathways responsible for controlling milk protein synthesis directly or indirectly (possibly through changes in IGF system as suggested by McGuire *et al.* 1995, Griinari *et al.* 1997), or alternatively, if the milk protein response is an adaptation of these animals to the change in the availability of nutrients caused by the constant elevation in circulating insulin concentrations.

However, not all studies using the HEC have realised an increase in milk protein (Tesseraurd *et al.* 1992, Annen *et al.* 1998). The reason for the variation in the milk protein response to the HEC is not known and further research is needed to understand the mechanisms involved.

The increase in milk protein production with the HEC technique has been demonstrated in concentrate-fed animals fed to requirements but to date it has not been applied in pasture-fed ruminants. In pasture-based systems, animals have different feeding regimes and the composition of the diets varies compared to concentrate-fed animals. It is recognised that while pasture generally supplies adequate dietary crude protein, it is often lacking in energy. In particular, ryegrass/white clover pastures are often high in crude protein and neutral detergent fibre and low in non-structural carbohydrate when compared to overseas (e.g. NRC) recommendations for lactating dairy cows (Carruthers *et al.* 1996).

Ulyatt (1997) reviewed the small number of experiments reported in the literature tha measured the response of pasture-fed ruminants to protein supplementation. As with concentrate-fed animals, the response to protein and/or amino acid (AA) supplementation was extremely variable. It was concluded that there was a production response only if the animal was limited in protein and/or AA supply, or if there was surplus energy available at the tissue level. Similar variability in response may be expected from pasture-fed ruminants exposed to changes in insulin status induced by the HEC.

Since supplementation of the diet with extra protein in both pasture and concentrate systems is not a reliable method of increasing the yield of milk protein, new approaches are needed to understand milk protein production, starting with a better understanding of AA metabolism. Firstly, mechanisms that control the partitioning of AA to the mammary gland for milk protein synthesis must be defined. Secondly, in order to manipulate the production of individual proteins by the lactating mammary gland a detailed understanding of the metabolic processes of milk production is required. The objective of the research conducted for this thesis was to contribute to this understanding, with a particular emphasis on insulin given its role in energy and protein metabolism in all mammals.

In trying to determine the role of insulin in milk protein synthesis, it is necessary to consider that insulin has roles (and possibly different roles), in both mammary and extramammary tissues that contribute to the regulation of milk protein synthesis. The following sections of this literature review will examine areas that insulin could influence that may a) change the partitioning of nutrients away from peripheral tissues to preferentially supply the mammary gland, and b) pathways that could be directly altered in the mammary gland.

1.2 The role of insulin during lactation

Insulin, as one of the major anabolic hormones of the body, is responsible for stimulating the uptake of nutrients by muscle and adipose tissue (reviewed by Hart 1983, Vernon 1989, Grizard *et al.* 1995, Grizard *et al.* 1999). In summary, the main processes regulated by insulin include promotion of lipid synthesis and inhibition of lipolysis in adipose tissue, promotion of protein synthesis and inhibition of proteolysis in muscle tissue, stimulation of glucose uptake by muscle and inhibition of gluconeogenesis and ketogenesis in the liver. Insulin can potentially affect the milk precursor and energy substrate supply to the mammary gland through these processes, many of which are diverting nutrients away from the mammary gland towards the peripheral tissues and this may seem to conflict with a role in regulating milk protein synthesis.

However, the effects of insulin are modified with the onset of lactation. It is important to recognise that the sensitivity of peripheral tissues to insulin changes with the physiological condition of the animal e.g. pregnancy, lactating or non-lactating (dry). In addition, these adaptations change over the course of lactation. It appears that the adaptations described in the following section are a co-ordinated response to blunt the effects of insulin on whole body metabolism, thereby preferentially supplying nutrients to the mammary gland.

During lactation, particularly early lactation, insulin concentrations are low in ewes (Vernon & Flint 1983), dairy cows (Jenny *et al.* 1974, Lomax *et al.* 1979) and goats (Debras *et al.* 1989) when compared to dry animals. These low insulin concentrations

have been attributed to negative energy balance, particularly in early lactation, and this would allow mobilisation of endogenous energy sources in the form of amino acids and NEFA from muscle and adipose tissue respectively. Circulating insulin concentrations increase as lactation progresses, the animal moves increasingly into positive energy balance and milk yields fall (Cowie 1980).

The ability of insulin to lower blood glucose concentrations is impaired during lactation, and the clearance of insulin from the circulation is faster. This decreased insulin responsiveness to glucose and increased metabolic clearance rate has been reported in lactating goats (Grizard *et al.* 1988, Debras *et al.* 1989), ewes (Faulkner & Pollock 1990) and dairy cows (Sano *et al.* 1993). In addition, Debras *et al.* (1989) observed that inhibition of hepatic glucose synthesis by insulin is lower in early relative to late lactation.

The responsiveness and sensitivity of adipose tissue and muscle is impaired during lactation and this is commonly called the insulin resistance of lactation. These changes may alter nutrient uptake by adipose and muscle tissue, indirectly influencing metabolism of the mammary gland. For example, whole body, insulin stimulated, glucose utilisation is impaired in dairy cows (Jenny *et al.* 1974) while the ability of insulin to stimulate glucose uptake by hind limb in ewes is diminished during lactation (Vernon *et al.* 1985). As glucose transport into, and utilisation by, adipose tissue and skeletal muscle is reduced (Grizard *et al.* 1988, Debras *et al.* 1989), the supply of glucose to the mammary gland for milk production can be increased.

The mechanism that causes insulin resistance is unknown but it appears that some component involved in passage of the insulin-induced signal through the plasma membrane is altered during lactation. The work on resolving the molecular basis of insulin resistance in lactation has been reviewed by Vernon & Pond (1997). Adipocytes from sheep (Vernon *et al.* 1981) and cattle (Vernon *et al.* 1985) possess insulin receptors. However, during *in vitro* experiments, little response to insulin is shown during short incubations. Longer incubations, for 24 h or more, increase lipogenesis and also the rate of glucose utilisation. The difference in response does not seem to be related to the ability of the cells to bind insulin, as there is no change in the number or affinity of the receptor for insulin during lactation (Vernon *et al.* 1981, 1985, Wilson *et*

al. 1996, Travers *et al.* 1997). As with adipose tissue, there is no alteration in number or affinity of insulin receptors, in bovine (Metcalfe *et al.* 1991) or ovine (Wilson *et al.* 1996) skeletal muscle.

The current evidence suggests that as the number and affinity of receptors in adipocytes and muscle do not change during lactation the defect is downstream of the receptor. Travers *et al.* (1997) reported that the expression of the gene encoding acetyl-CoA carboxylase (ACC), the rate determining enzyme of the lipogenic pathway, is reduced in adipose tissue depots in lactation. In addition, total ACC enzyme activity is markedly reduced. When adipose tissue from lactating ewes was incubated with insulin, there was an increase in ACC mRNA, total enzyme activity and the proportion of enzyme in active state. However, it was between 32 and 48 hours before marked changes in these parameters were observed (Travers *et al.* 1997). This evidence suggests that the reduction in lipogenic capacity in ovine tissue during lactation is related to the repression of the ACC gene at the level of mRNA abundance, a slower rate of translation, and a smaller proportion of ACC in the active state.

In the mammary gland, insulin has been shown to be essential for ruminant mammary tissue development and cell maintenance *in vitro* (Collier *et al.* 1977, Baumrucker & Stemburger 1989). However, there is no evidence to suggest that insulin can directly stimulate mammary tissue to increase milk protein output during established lactation *in vivo*. Mackle *et al.* (2000b) administered insulin directly into the mammary gland via the teat but this did not result in any evidence of increased protein synthesis or an increase in milk protein yield. However, while this paper justified the use of this method, it may not be the most effective method to determine a direct effect on secretory cells in the mammary gland.

1.3 Insulin and milk protein production

Early studies indicated that the role of insulin in milk protein synthesis in the lactating mammary gland was hard to define. In these studies insulin was administered in bolus injections, which resulted in a depression in plasma glucose concentration, milk yield (Kronfeld *et al.* 1963, Schmidt 1966) and milk lactose percentage (Schmidt 1966). However, milk fat and protein percentages, but not actual yields, increased (Schmidt

1966). Milk yield was restored to pre-treatment values with the administration of glucose (along with continuing insulin administration) (Kronfeld *et al.* 1963). The decrease in milk yield appeared to have resulted from induced hypoglycaemia and was not a direct result of insulin action on the mammary gland. Insulin alone caused a significant increase in milk protein percentage in comparison to control and insulin plus glucose infusion values (Schmidt 1966). Schmidt concluded that the increases in milk protein and fat percentages were due to a stimulatory effect of insulin on secretion rate of protein and fat as the increases in both could not be explained entirely by the decrease in milk volume produced.

Later studies concentrated primarily on the effect on lactose and milk yield only, from which it was concluded that the lactating ruminant mammary gland is relatively insensitive to the actions of insulin on glucose metabolism. Insulin has been shown to have no acute effects on the rates of glucose uptake or milk synthesis in lactating ruminants (Hove 1978, Laarveld *et al.* 1981, Tesseraud *et al.* 1992). Infusing lactating goats and dairy cows with glucose and insulin caused an increase in circulating insulin concentrations but no subsequent change in plasma glucose arterio-venous differences or extraction rates (Hove 1978, Laarveld *et al.* 1981, Tesseraud *et al.* 1992). However, these studies were of short duration (2-10 h), and did not allow any chronic effects of insulin administration to be demonstrated. Nevertheless, the results would appear to indicate there was no effect of insulin administration on mammary gland glucose uptake or lactose synthesis. Therefore, short-term administration of insulin that is known to alter glucose metabolism in other tissues, such as muscle, does not seem to alter mammary glucose metabolism, milk yield or the yield of milk components. In addition, it appears glucose turnover in the mammary gland is independent of changes in plasma insulin concentrations. This is in agreement with Kronfeld (1963), who also reported that large variations in plasma insulin concentrations might occur without observable changes in mammary consumption of glucose. This is possibly an adaptation to keep the supply of glucose to the mammary gland as constant as possible despite the fluctuations in insulin concentrations that occur in response to changes in nutrient flow from the alimentary tract.

However, recent studies using the HEC have demonstrated that, under these conditions, there is an increase in milk and milk protein production in animals fed to requirement.

The HEC is a technique that uses simultaneous infusions of insulin and glucose that allows plasma insulin to be raised but prevents the development of hypoglycaemia. A list of recent HEC studies and the effects on milk and milk protein production are shown in Table 1-1. As the clamp was maintained for 3.5 to 4 days, this allows the chronic effects of insulin infusion to be examined. In all studies where there was a milk protein response, the increase in milk protein production was not acute but gradual over the duration of the HEC.

The study by McGuire *et al.* (1995) demonstrated that manipulating insulin concentrations via the HEC in concentrate fed cows increased milk protein yield by 7% (Table 1-1) without changing milk yield. However, because of the decrease in circulating concentrations of AA in this study it was thought that the milk protein response was limited by AA supply to the mammary gland. Subsequent studies have shown that when insulin status was manipulated by the HEC in conjunction with supplemental dietary protein, milk protein yield was substantially increased. Griinari *et al.* (1997) administered additional protein post-ruminally in the form of casein and when the cows were subjected to the HEC, milk protein yield increased by 230g/d (28% increase over baseline). While milk protein concentration was increased (Table 1-1), protein composition was not altered. Because of the large decreases seen in circulating BCAA, Annen *et al.* (1998) supplemented cows with additional BCAA during the HEC. In contrast to the other studies in the table, there was no milk protein response with the HEC alone or HEC plus BCAA.

However, Mackle *et al.* (1999, 2000a) supplemented cows with casein plus additional BCAA during a HEC. This resulted in an increase in milk protein yield of 25% as a result of a 15% increase in milk yield and an 11 % increase in protein concentration but there was no effect of the additional BCAA supplementation.

In lactating goats, the HEC with and without simultaneous intravenous infusion of an AA mixture increased milk protein yield (Table 1-1) despite milk protein concentration being significantly reduced (Bequette *et al.* 2001). In this study, and that by Mackle *et al.* (2000a) there was also significant increases in milk yield, which were not seen in previous experiments.

Table 1-1 Comparison of changes in milk protein yield (kg/d) and composition (%) in hyperinsulinaemic euglycaemic clamp (HEC) studies.

Species	No supplementation		Supplementation		Reference
	Control	Control + HEC	Casein/AA	Casein/AA + HEC	
Milk protein yield (kg/d)					
Dairy cows	0.98	1.05	n/a	n/a	McGuire *et al.* (1995)
Dairy cows	0.81	0.89	0.84	1.04	Griinari *et al.* (1997a)
Dairy cows[1]	0.885	0.878	0.83	0.804	Annen *et al.* (1998)
Dairy cows[2]	0.867[a]	0.995[b]	0.895[a]	1.08[c]	Mackle *et al.* (2000a)
Dairy goats	0.097	0.104	0.099	0.110	Bequette *et al.* (2001)
Protein Concentration (%)					
Dairy cows	3.04	3.14	n/a	n/a	McGuire *et al.* (1995)
Dairy cows	3.11	3.15	3.14	3.44	Griinari *et al.* (1997a)
Dairy cows	3.29	3.25	3.18	3.29	Annen *et al.* (1998)
Dairy cows[2]	3.29[a]	3.52[b]	3.52[a]	3.66[b]	Mackle *et al.* (2000a)
Dairy goats	3.41	3.21	3.42	3.25	Bequette *et al.* (2001)

[1] Yields calculated from milk yield x crude protein concentration.
[2] Only study where statistical difference between means were tested.
n/a = not applicable as no supplementation fed.
[a, b, c] LSMeans with different superscripts are significantly different at $P<0.05$.

Different supplemental protein sources and statistical analyses were used to examine the role of insulin (using the HEC) in milk protein production (Table 1-1). I have attempted to look as these studies in terms of HEC (insulin) alone and HEC x protein interaction. All studies were reported as showing significant effects of insulin but this is sometimes difficult to see in the values presented as, only in the study of Mackle *et al.* (2000a) were the means of treatments tested to see if they were significantly different. However, in studies where there was a milk protein response reported, the HEC alone appears to stimulate an increase in milk protein yield in the range of 7-15 % (McGuire *et al.* 1995, Mackle *et al.* 2000a, Bequette *et al.* 2001). The large yield response of 25-28 % results from an interaction between the HEC and supplemental protein in the form of casein (Griinari *et al.* 1997, Mackle *et al.* 2000a).

These results are remarkable when considered relative to the adaptations of whole body metabolism to meet the requirements of lactation, which primarily seem to blunt the effects of insulin, particularly in adipose and muscle tissues. It appears that if the glucose status of the animal is not compromised, insulin may have a role in regulating milk protein synthesis. However, not all studies of this type, either short (Tesseraud *et al.* 1992), or long term (Annen *et al.* 1998) have demonstrated a milk protein response. Therefore, it needs to be determined if the response to insulin seen in these studies is a direct effect of stimulating amino acid uptake and subsequent milk protein synthesis and secretion or is it an adaptation to the change in the nutritional environment induced by the HEC which may allow preferential nutrient partitioning to the mammary gland for milk protein production.

The following sections will review and discuss areas that insulin has the potential to alter in milk protein production through direct effects on the mammary gland or indirect effects on whole body protein metabolism.

1.4 Amino acid supply to the mammary gland

Amino acids, particularly plasma-free AA have been shown to be the major precursors for milk protein synthesis. It has previously been demonstrated in non-lactating and growing ruminants that administration of insulin results in decreased plasma AA concentrations (see Lobley 1992, 1993 for reviews). In lactating ruminants, the HEC and infusion of insulin alone have also decreased circulating concentrations of AA.

1.4.1 Changes in plasma amino acid concentrations in HEC studies

A short (2.5 h) HEC with simultaneous infusion of AA found 2 dose rates of insulin reduced plasma AA concentrations (Tesseraud *et al.* 1992). Despite the short time, arterial concentrations of some EAA (leucine, threonine, valine, isoleucine, tyrosine and histidine) and some NEAA (aspartic acid, serine, glutamic acid, glycine and citrulline) were significantly decreased.

In long term (3.5 – 4 d) experiments with the HEC in which circulating concentrations of plasma EAA were measured (McGuire et *al.* 1995, Griinari *et al.* 1997a, Mackle *et*

al. 1999), concentrations of total EAA decreased from 33-60%. The decrease in BCAA alone ranged from 41-73%.

The decreases in plasma AA concentrations in these studies using the HEC in lactating cows were associated with increases in milk protein yield that could be interpreted as indicating that the decrease in AA concentrations was because of greater demand by the mammary gland. In addition, in recent HEC studies the arterial supply of EAA to the mammary gland has been reduced in lactating cows (Mackle *et al.* 2000a) and goats (Bequette *et al.* 2001). Concentrations of most EAA were reduced except for threonine and tyrosine (Mackle *et al.* 2000a) and phenylalanine and tyrosine (Bequette *et al.* 2001). In both these studies the concentrations of BCAA were reduced on average by 55% and the total supply of AA to the gland was reduced by the HEC.

As stated previously, similar decreases in AA concentrations have also been seen in non-lactating animals. Therefore, there is no direct evidence to suggest that insulin acts directly on the mammary gland to stimulate AA uptake. In fact, the decrease in arterial concentrations seen in the studies by Mackle *et al.* (2000a) and Bequette *et al.* (2001) indicates the decrease may be caused by the effect of insulin in other tissues, such as liver and splanchnic tissue, and muscle.

1.4.2 Effects of insulin on splanchnic tissue and liver metabolism

The mechanisms whereby the administration of insulin reduces plasma concentration of AA are unclear but may involve changes in liver and muscle metabolism. Determining the effects of insulin in tissues such as liver and muscle in ruminants and non-ruminants is difficult because of the cycle of protein synthesis and degradation.

Lobley (1992) suggested that insulin decreases plasma AA concentrations by stimulating their net uptake by the peripheral tissues, as insulin does not alter hepatic removal of AA (Brockman *et al.* 1975). However, Biolo & Wolfe (1993) suggested that under HEC conditions, particularly if there is no protein supplementation, the plasma as well as muscle free AA concentration drops because of reduced AA release from splanchnic tissue.

As a result of the range of its possible effects, the role of insulin on AA uptake and metabolism in muscle tissue is discussed in more detail in the following section, before considering its role in AA uptake and metabolism in the mammary gland in section 1.5.1.

1.4.3 Protein metabolism in skeletal muscle and how this changes during lactation

Muscle is the largest protein pool in the body and is important in lactating ruminants as a protein reserve of AA for glucose production and milk protein synthesis. Experiments using isotopically labelled AA have monitored protein turnover (in terms of synthesis and degradation) and the metabolic fate of AA in ruminants and non-ruminants (reviewed by Harris & Lobley 1991, Lobley 1992, 1993, 1994) on a whole body basis and across tissue beds such as muscle and the mammary gland. Models have been developed that allow the calculation of whole body fluxes of individual AA (Harris *et al.* 1992) that have then been used to measure fluxes across tissues such as the mammary gland (Bequette *et al.* 1996a,b). Other models have been developed that have been used across skeletal muscle beds such as the hind-limb (Oddy *et al.* 1986) and also across the mammary gland (Oddy *et al.* 1988).

During lactation, there is often a period of negative N balance when rate of muscle protein synthesis is lower than the rate of degradation (review; Kelly *et al.* 1991), resulting in a net loss of skeletal muscle. It would appear that the muscle loss is due to an increase in the rate of protein degradation with little or no change in protein synthesis. Vincent & Lindsay (1985) reported that lactation induced a 25% increase in protein synthesis rate but also a 30% increase in fractional degradation rate. Champredon *et al.* (1990) also reported that the loss of skeletal muscle in early lactation is because of both an increase in protein degradation and a decrease in protein synthesis in muscle. This is an adaptive mechanism that would decrease the use of AA by extramammary tissues, so that AA released from muscle could be used for protein synthesis in the mammary gland or gluconeogenesis in the liver.

Circulating insulin has an important role in this changing pattern of muscle metabolism during lactation. It is generally accepted that insulin promotes protein deposition in muscle by both inhibiting proteolysis and stimulating protein synthesis. However,

while insulin has been demonstrated to stimulate protein synthesis *in vitro*, it appears that *in vivo*, the ability of insulin infusion to stimulate muscle protein synthesis is only evident in young growing animals in post-absorptive state (e.g. Garlick & Grant 1988) and this decreases with age (review; Reeds *et al.* 2000). In ruminants, insulin infusion did not alter protein synthesis in the hind-limb of fed or fasted lambs (Oddy *et al.* 1987, Early *et al.* 1988a, 1988b) and indeed, in one study (Oddy *et al.* (1987) a decrease in protein degradation in the fasted condition was observed. This changing sensitivity of protein metabolism to exogenous insulin dependent upon the energy balance of the animal may reflect what happens in lactation, especially when lactating animals are in a negative energy balance.

The HEC technique and [^{14}C]-leucine tracer studies have been used to determine the role of insulin in muscle metabolism during lactation. In lactating goats, insulin failed to stimulate non-oxidative disposal of [^{14}C]-leucine, an estimate of whole body protein synthesis, even when combined with AA infusion (Tesseraud *et al.* 1993). In addition, insulin caused a dose-dependent inhibition of endogenous leucine appearance, which is an estimate of whole body degradation of protein and this effect was greater in early lactation. Tauveron *et al.* (1994) also reported that under HEC conditions and using a large flooding dose of valine, there was no stimulatory effect of insulin on muscle protein synthesis in lactating goats.

The inhibition of whole body proteolysis by insulin is now well recognised. Recent studies demonstrate that insulin acts in skeletal muscle on the ATP-ubiquitin-proteasome pathway by decreasing ubiquitin expression (review; Grizard *et al.* 1999). That the antiproteolytic effect of insulin is improved during lactation may prevent excessive muscle wasting and would conserve body proteins, AA and gluconeogenic substrates which in turn would potentially be available for milk protein synthesis.

1.4.3.1 The role of insulin in amino acid oxidation in muscle

Information on the effect of insulin on AA metabolism in muscle comes from studies on BCAA. These AA acids may be important in milk protein synthesis because over 50% of BCAA escape hepatic removal (gluconeogenesis) (Harris & Lobley 1991). Branch-chain AA have a direct role in the synthesis of milk protein as precursors and an indirect

role in supplying amino groups for the synthesis of NEAA in the mammary gland. This is demonstrated by the difference between mammary uptake and output for individual AA. For example, arginine and BCAA are taken up in excess of their output in milk protein, whereas several NEAA are taken up in insufficient quantities to account for their output in milk protein (Mepham *et al.* 1982).

One aspect of AA metabolism in the body that could potentially be changed by variations in insulin concentration is AA oxidation. It is not known whether oxidation is a mechanism solely for the removal of AA surplus to requirements, or if regulatory intermediary processes require the catabolism of AA. It may be that insulin by regulating this process in muscle cells affects the supply of AA for milk protein synthesis.

The AA of muscle protein continually turn over, such that protein degradation provides a mobile source of AA precursors for protein synthesis throughout the body. If lactation creates a demand for AA that are limiting for milk protein synthesis, this may require mobilisation of proteins, which results in an excess of other released AA, which are then oxidised. Insulin may have a role in preventing this oxidation. If 30-40% of oxidation of BCAA occurs in peripheral tissues (Lobley 1993), and addition of insulin suppresses this oxidation, then these AA may become available to the gland. It appears that muscle uptake of AA continues in the presence of insulin but after degradation, it is the oxidation process that is altered. The BCAA have a unique catabolic sequence where there is reversible transamination to form the appropriate keto acid (e.g. leucine is transaminated to α-keto isocaproate). This is followed by irreversible decarboxylation by the enzyme branch chain keto acid dehydrogenase. Insulin can alter the phosphorylation state of the branch chain keto acid dehydrogenase (Block *et al.* 1987), which controls the production of the keto acid. Insulin increases the rate of phosphorylation, which inactivates the enzyme, and suppresses the production of the keto acid. Therefore, administration of insulin may reduce the amount of BCAA that is converted to the keto acid and then oxidised to CO_2. While this pathway is specific for the BCAA, it may also influence the oxidation rate of other AA.

Decreased oxidation would release mobilised AA back into the blood stream for utilisation by other tissues, and Brockman *et al.* (1975) reported that the decrease in

plasma AA during insulin and glucose infusions were not associated with increased utilisation of AA by the liver. Therefore, there is the potential for increased uptake by the mammary gland. When estimates of peripheral metabolism are scaled to whole body, oxidation by muscle accounts for 36-65% of the AA metabolised in well-fed ruminants (Harris & Lobley 1991). Under fasting or restricted nutrition, the rate of oxidation decreases but the proportional contribution is unchanged for sheep. Therefore, if AA oxidation is reduced, these AA are potentially available for uptake by the mammary gland.

1.4.4 Mammary blood flow

Mammary blood flow is crucial component of substrate supply to the gland for milk synthesis. This is demonstrated in the high correlation shown between mammary blood flow and milk yield (Linzell 1974).

Early work on mammary blood flow (reviewed by Davis and Collier 1985) showed that mammary blood flow was under nutritional and hormonal control and was reduced by restricted feeding or increased by administration of bovine somatotropin (bST) or thyroxine. The regulation of mammary blood flow in response to substrate availability was demonstrated by Bequette *et al.* (2000) where goats fed a histidine-deficient diet increased mammary blood flow (by 50%) and mammary histidine extraction rates (from 17-90%). However, the signal or trigger to the mammary gland has not been identified.

Later work (reviewed by Prosser *et al.* 1996) shows the mammary gland can regulate its own blood supply and there are local factors that regulate mammary blood flow through effects in the mammary microvasculature. Here, a number of locally produced vasoactive compounds such as parathyroid hormone-related protein, IGF-1, prostacyclin, nitric oxide and endothelinin can alter blood flow (Prosser *et al.* 1996). These compounds potentially provide the mechanisms by which the mammary gland can control its own blood supply and thereby regulate precursor supply to meet the requirements for milk synthesis.

It has been suggested that changes in concentrations of insulin-like growth factor (IGF) 1 and 2 (IGF-1, IGF-2) may be signals to increase mammary blood flow. That IGF may

be important comes from studies where cows treated with bST have increased concentration of IGF-1 and mammary blood flow (Davis *et al.* 1988). It was thought that IGF mediates the action of bST in the mammary gland (reviewed by Cohick 1998) although there is no evidence of specific action for this. However, arterial infusion of IGF-I into the mammary gland has resulted in increased milk yield in some studies (Prosser *et al.* 1990, Prosser & Davis 1992) but not others (Davis *et al.* 1989). Certainly IGF-1 is a potent mammary vasodilator (Prosser & Davis, 1992) but this effect was insufficient to increase yield in circumstances where exogenous bST was effective (Davis *et al.* 1989). Further work is required to understand the mechanism of action of bST and the role (if any) of IGF-1.

It is not clear if insulin alone can increase mammary blood flow. Hove (1978) reported an increase in mammary blood flow in goats infused with insulin for 10 h, while infusing glucose at a fixed rate to maintain euglycaemia. Similarly, mammary blood flow increased on average by 42% in association with increased milk yield in 2 recent HEC studies (Mackle *et al.*2000a, Bequette *et al.*2001). However, mammary blood flow was reduced as a result of a close arterial infusion to the gland (Metcalf *et al.* 1991), although in this study euglycaemia was not maintained.

Plasma concentrations of IGF-1 are also seen to increase with the amplified insulin concentrations seen in HEC studies (McGuire *et al.* 1995, Griinari *et al.* 1997; Mackle *et al.* 1999, 2000a; Bequette *et al.* 2001). However, there is no direct evidence for a role of IGF-1 in increasing mammary blood flow or the milk protein response seen in these studies.

The regulation of mammary blood flow is complex and at present, there is no clear indication of a direct or indirect role for insulin. However, if mammary blood flow is increased under HEC conditions, this is one way of increasing precursor supply for milk protein synthesis.

1.4.4.1 Measuring mammary blood flow

Mammary blood flow can be measured using different methods (reviewed by Linzell 1974, Lescoat *et al.* 1996) and there is a range of flow values for lactating ruminants

that have been published and these are subject to the method used to measure blood flow, age, stage of lactation, posture and species. There is considerable debate over the accuracy of different methods of measuring mammary blood flow.

Two of the more common methods used in lactating ruminants are direct measurement using transit time flow probes and indirect estimation by the Fick principle using A-V concentration differences of methionine and/or phenylalanine+tyrosine. Some of the problems associated with using transit time blood flow probes include correct placement of the probe around pudic artery during invasive surgery (Gorewit *et al.* 1989). If incorrectly placed, the probes can underestimate flow. Farr *et al.* (2000) reported that in lactating goats, the mammary blood flow estimated by an indicator-dilution technique was 1.5 times larger than that recorded by the transit time probes. In addition, probes can malfunction following chronic implantation.

The use of A-V concentration difference and the Fick principle has been applied to AA for which the ratio of uptake by the mammary gland is considered to be approximately 1:1, such as the group 1 EAA methionine, lysine, phenylalanine+tyrosine (Davis *et al.* 1988). While a comparison of estimates of mammary blood flow using electromagnetic blood flow probes and phenylalanine+tyrosine showed no significant difference (Davis *et al.* 1988), this assumes there is no alternative supply of AA to the mammary gland. If erythrocytes or peptides contribute to mammary uptake of AA, mammary blood flow would be over-estimated. It has been demonstrated that both erythrocytes and peptide bound sources of methionine, lysine, phenylalanine and tyrosine are used by the lactating mammary gland. Wang *et al.* (1996) demonstrated *in vitro* that peptide bound methionine could be used as a source of methionine for milk protein synthesis by lactating mouse mammary tissue. This is supported by *in vivo* data presented by Pethick & Lindsay (1982) from a study with lactating ewes.

Even though it has been demonstrated that phenylalanine and tyrosine are not metabolised by mammary gland (Verbeke *et al.* 1972), it has also been demonstrated that mammary uptake of phenylalanine and tyrosine is not sufficient for that secreted in milk protein. Pacheco-Rios *et al.* (1999) demonstrated that in pasture-fed dairy cows, uptake of lysine, phenylalanine and tyrosine from plasma was insufficient for their output in milk protein. However, when whole blood was analysed in that study, the

contribution of erythrocytes to the uptake of lysine, phenylalanine and tyrosine was seen to be between 5-15% and this was sufficient to account for the output of those AA in milk protein. In lactating goats, Bequette *et al.* (1999) showed that between 5-25% of the supply of lysine, methionine, phenylalanine and tyrosine for casein synthesis came from vascular peptides. These results would suggest that the Fick principle methods may slightly underestimate flow.

The previous sections demonstrate some of the limitations to the different methods of measuring blood flow and the researcher must choose the method that is most pertinent, while acknowledging the limitations. Most methods are adequate for determining relative shifts in blood flow, but if determination of actual blood flow is required, then selection of a method becomes more critical.

1.5 Amino acid uptake by the mammary gland.

1.5.1 Uptake of AA by the mammary gland

There are a number of mechanisms that facilitate the movement of AA across the cell membrane for milk protein synthesis. The cell membrane is predominantly lipid in composition, which effectively prevents entry of the hydrophilic AA by simple diffusion. Therefore, transport across the basal membrane depends on carrier systems (Mepham 1982).

It is suggested that insulin can affect the uptake of amino acids by the mammary gland. A close arterial infusion of insulin plus AA into the mammary gland increased the uptake of threonine, phenylalanine, methionine, leucine and lysine (Metcalf *et al.* 1991). In this study there was a small but non-significant increase in milk protein yield and milk yield, which indicates some of the infused amino acids were used as an energy source. This would appear to indicate that a concurrent increase in energy supply might be required to increase milk protein synthesis when using insulin.

In the HEC studies where the decreases in circulating concentrations of AA were associated with an increased milk protein production (McGuire *et al.* 1995, Griinari *et al.* 1997), it was hypothesised that this decrease was caused by increased uptake of AA by the mammary gland. However, in the only HEC study where AA uptakes by the

mammary gland were published (Bequette *et al.* 2001), the mammary uptake of histidine, isoleucine, leucine, valine, lysine, threonine, glutamate, glycine and serine were significantly reduced, whereas the uptake of alanine was increased.

At present the evidence for a role for insulin in stimulating AA uptake by the mammary gland is inconclusive. However, *in vitro* studies have provided some evidence that insulin could influence AA transport systems, and this is considered in the following section.

1.5.2 Amino acid transport systems

Amino acids have specific transport systems and within that system, transport specificity and competition determine overall flux (reviewed by Baumrucker 1985). However, considerable overlap of transport of certain AA can occur, especially in the neutral systems (reviews: Baumrucker 1985, Guidotti *et al.* 1978). The three main systems (A, ASC and L) transport neutral AA with an extensive overlap of substrate specificity (reviewed by Guidotti *et al.* 1978). For example, the A system may transport most AA. These systems and the AA they transport are described in Table 1-2.

A feature of the AA transport systems (shown in Table 1-2) is the similar extraction percentages for the AA when grouped according to their transport system despite considerable variation in their plasma concentrations (Baumrucker 1985). The extraction percentage for methionine, which is transported by both the ASC and L systems, is very high.

Table 1-2 Possible amino acid transport systems in bovine mammary tissue and (% extraction) derived from A-V studies (adapted from Baumrucker 1985).

System		Principle amino acids transported with (% extraction)
Neutral	A	Alanine (12), Glycine, Proline (21), Methionine (58)*
	ASC	Cysteine, Serine (29), Threonine (30), Methionine*
	L	Valine (24), Leucine (38), Isoleucine (40), Phenylalanine (40), Tyrosine (42)
Cationic	Y^+	Lysine (55), Arginine (53) , Ornithine (29)
Anionic		Aspartate (18), Glutamate
N		Histidine (30), Glycine (24), Asparagine (29)

* = combined total for A and ASC systems

Most transport systems operate well below saturation (Baumrucker 1985) which suggests that additional supply of AA via nutrition or blood flow, would lead to increased uptake of AA by the mammary gland, which could then result in an increase in milk protein synthesis. This is in agreement with reports of post-rumen protein infusions (Clark *et al.* 1977, Guinard *et al.* 1994) increasing milk protein yields. In addition, it has been demonstrated with the mammary cationic system that increasing circulating arginine and lysine concentrations results in greater cellular concentrations (Baumrucker 1984) and which are potentially available for increased milk protein synthesis.

Evidence for insulin stimulating AA uptake through effects on transport systems has been provided by *in vitro* experiments with mammary cells. Insulin stimulated the uptake of cycloleucine by bovine mammary acini (Park *et al.* 1979) and the uptake of α-amino-isobutytic acid (AIB) by mouse mammary explants (Friedberg *et al.* 1970, Oka *et al.* 1974) indicating that system A for AA transport is sensitive to insulin in the mammary gland across species. Insulin is believed to stimulate an increase in the synthesis of new carrier proteins that facilitates the uptake of the AA (Guidotti *et al.* 1978).

To date there is no evidence to suggest that changes in AA transport systems were responsible for the milk protein response seen in recent HEC experiments. However, as transport systems operate well below saturation, there is the potential for insulin to increase mammary AA uptake through inducing changes in the activity of transport systems.

1.6 The role of amino acids in milk protein synthesis.

The major milk proteins synthesised by the lactating ruminant mammary gland are the casein and whey proteins. The caseins are made up of the α-CN, κ-CN, β-CN, γ-CN. The major whey proteins are α-lactalbumin (α-LG) and β-lactoglobulin (β-LG). The remaining proteins are predominantly derived from the blood serum such as blood serum albumin (BSA) and the immunoglobulins (IgG).

All the epithelial cells lining the alveoli and ducts that consist of a single layer of cells in the mammary gland are thought to contribute to milk protein synthesis but it is not known if all cells synthesise and secrete milk proteins constantly or in a phasic fashion. Molenaar *et al.* (1992) examined mRNA expression of α-LG, α-S_1-CN and lactoferrin in non-lactating and lactating mammary cells. Results showed that there were 2 classes of alveoli that were classed as fatty (non-active) or non-fatty (active). Gene expression of α-LG and α-S_1-CN were high in the non-fatty alveoli, which suggested that there may the potential to increase the cells that are actively lactating. There is no indication at present how hormones such as insulin could effect the regulation of this process.

1.6.1 Mammary tissue protein synthesis

Protein synthesis in the mammary gland consists of milk proteins that are secreted and constitutive proteins such as structural proteins (which contribute to tissue mass) and enzymes. In lactating animals, it contributes a large proportion of whole-body protein synthesis compared to dry animals. In lactating goats, mammary tissue protein synthesis (absolute rate of protein synthesis plus net export of milk protein) was calculated to be 46 % of the estimated whole body protein synthesis rate (Champredon *et al.* 1990).

Milk proteins are synthesised at a greater rate than constitutive protein mass of the mammary gland. While there is no direct measurement of synthesis rates of constitutive vs milk proteins, the ratio of milk protein secreted (g/d) to mammary tissue mass (g) was 0.50 in lactating goats (Champredon *et al.* 1990), and 0.45 in cows (Bequette *et al.* 1998).

Amino acids labelled with various isotopes have been used to examine the precursor pools for casein synthesis. ^{13}C-AA infusions were used to compare the rates of labelling of free AA in blood and plasma with that secreted in milk proteins in dairy cows (Bequette *et al.* 1996a) and goats (Bequette *et al.* 1997). Free AA in the blood and plasma reached isotopic plateau between 1-3 hours of commencing the infusion compared to 10-13 hours to reach plateau in milk casein and whey proteins. Appearance of the isotope in the milk in less than 1 hour after commencing the infusion indicated that the interval between synthesis and secretion is short, and storage pools are small.

The overall slow rate of the ^{13}C-AA in milk proteins to reach plateau suggests a substantial contribution of intracellular degradation products from the turnover of milk and constitutive proteins into the precursor pool for milk protein synthesis. Oddy *et al.* (1988) reported that in the mammary gland of the lactating goat the rate of total protein synthesis in the mammary gland was greater than milk protein secretion, indicating that there is substantial synthesis and degradation of protein in the mammary gland. An estimate for the partitioning of protein synthesis between tissue (constitutive) and milk protein suggested that one third of synthesised milk proteins are degraded (Oddy *et al.* 1988). In lactating dairy cows, mammary gland protein synthesis was always greater (by 20-59%) than milk protein output (Bequette *et al.* 1996a), suggesting substantial synthesis of non-milk proteins and/or turnover of synthesised milk protein.

The extent to which the turnover of milk and constitutive proteins contribute to total protein turnover in the gland and whether such degradation is obligatory remains to be determined. It is suggested that this protein turnover in the mammary gland acts as a pool or buffer that ensures the constant availability of AA for milk protein synthesis during short periods of deficiency.

1.6.2 The role of amino acids in milk protein synthesis.

There are three possible sources of precursors in the blood for milk protein synthesis - plasma proteins, peptides and free AA. It is generally accepted that free AA absorbed by the mammary gland provide most of the nitrogen required for synthesis of milk protein. Studies summarised by Mepham *et al.* (1982) using isolated perfused mammary gland preparations, show that the percentage of the individual AA residues in the milk protein that were derived from a plasma AA precursor was 86% for methionine, 85% for threonine, 96% for phenylalanine, 96% for valine and 84% for arginine. More recent experiments (Backwell *et al.* 1994, 1996) have demonstrated directly and indirectly that the lactating goat mammary gland has the ability to utilise peptide AA. However, the amount of peptide AA that the mammary gland does actually utilise remains to be adequately quantified.

To exploit the mammary potential for milk protein synthesis and secretion, an optimal supply of AA is required. Mepham (1982) recommended that mammary uptake of AA depends on 1) arterial concentration and 2) blood flow - together these factors determine the quantity of AA delivered to the glands per unit time, and 3) the extraction rate by which AA are absorbed into the secretory cells (Mepham 1982). As a result of this, the elevation of arterial AA concentrations by various methods are seen as possible approaches to increasing AA supply to the mammary gland milk protein yield. Even now, most studies appear to have concentrated on aspects of points 1 and 2, and little has been quantitatively determined about point 3.

Once inside the cell, amino acids may:

1) undergo RNA directed polymerisation (i.e. protein synthesis) to form milk proteins, which are subsequently secreted by exocytosis
2) retained in cells in the form of structural proteins or enzymes
3) enter metabolic reactions yielding CO_2 , urea, polyamines and NEAA
4) pass unchanged into milk, blood and lymph.

The first option is quantitatively the major route for AA in the mammary gland, but this is not the case for all EAA, especially under certain conditions of substrate supply.

To quantitatively determine the utilisation of amino acids by the mammary gland, A-V differences and blood flow estimates are compared with the output of amino acids in milk protein. Generally, it is now accepted that while for some AA, uptake is balanced with output in milk protein, for others, uptake is inadequate while some are absorbed in excess (Bickerstaffe *et al.* 1974, Mepham and Linzell 1974, Davis and Mepham 1976). The AA have been summarised in groups that can be described as:

a) NEAA group. The uptake of NEAA vary considerably from hour to hour, but are, on average, inadequate to provide the corresponding residues in milk-specific proteins.

b) Group 1. Certain EAA (histidine, methionine, phenylalanine, tyrosine and tryptophan) are quantitatively transferred to milk protein.

c) Group 2. The remaining EAA (arginine, leucine, isoleucine, lysine, threonine and valine) are taken up in excess in relation to their output in milk protein. This difference is greatest for arginine and the BCAA. The excess are transaminated providing amino groups for the synthesis of NEAA while the carbon skeletons are oxidised to provide energy or enter other general metabolic pathways such as the gluconeogenesis pathway.

Studies with isotopically labelled AA have demonstrated the interconversion of AA within the mammary gland. For example, infusions of ^{14}C-arginine into the perfused goat mammary gland demonstrated that excess arginine was hydrolysed to ornithine and urea, with the arginine carbon being used in the mammary synthesis of milk protein proline (Mepham and Linzell 1967). Ornithine produced as a result of arginine hydrolysis is used (via glutamic γ-semialdehyde) as a carbon precursor of proline, and by irreversible transamination involving the δ-amino group it acts as a carbon precursor of several other NEAA, including glutamate, alanine and serine (Mepham *et al.* 1982).

The catabolism of BCAA has been investigated in bovine mammary slices (Wohlt *et al.* 1977) and perfused guinea pig mammary gland (Davis and Mepham 1976) experiments. Oxidation of labelled valine, leucine, and isoleucine has been shown by the identification of administered ^{14}C in CO_2. It would appear that BCAA undergo an initial transamination reaction and the carbon skeleton is catabolised by pathways that yield TCA cycle intermediates via acetyl CoA and succinyl CoA (Davis and Mepham 1976). The transamination reactions also appear to be reversible as there was a higher

recovery of ^{14}C of free valine, leucine and isoleucine when α-keto acids were included in the incubation medium (Wohlt *et al.* 1977). These experiments also demonstrate the use of BCAA for NEAA synthesis. ^{14}C was recovered in glutamine and aspartate in the bovine mammary tissue incubations (Wohlt *et al.* 1977) and in glutamate, glutamine and proline from leucine oxidation in the guinea pig mammary gland (Davis and Mepham 1976).

As with muscle, the potential for insulin to reduce BCAA oxidation may provide increased AA supply for milk protein synthesis within the mammary gland. Bequette *et al.* (2001) reported leucine oxidation was reduced by 40 % during the HEC and this was associated with an increase in milk yield and milk protein yield.

1.7 Conclusion

From the previous sections it appears there is potential for insulin to be able to influence the regulation of milk protein synthesis and subsequent production in the mammary gland as has been demonstrated by the Cornell studies. While the milk protein response reported by McGuire *et al.* (1995) was small (7%) there was no supplemental protein supplied. Whereas, the significantly large (25-28%) increases were reported when extra protein was supplied via the abomasum (Griinari *et al.* 1997, Mackle *et al.* 1999, 2000a, Bequette *et al.* 2001). Even then, the milk protein response develops slowly, over a period of 3-4 days. Furthermore, attempts to replicate the Cornell data have been unsuccessful (Annen *et al.* 1998) as there was no milk protein response to the HEC alone or the HEC with additional BCAA supplied.

Previous experiments have provided few insights into how the milk protein response to insulin was achieved. It is unknown whether direct effects of insulin on the mammary gland mediate the response or if what occurs is a result of the effects of insulin on metabolism in peripheral tissues such as muscle and adipose tissue with consequences to mammary substrate supply. In addition, when potential changes as a result of secondary endocrine mediators are considered, the picture becomes even more complicated.

Given the information above, the objectives of this research were designed to provide information on the potential for the manipulation of the insulin axis to promote milk protein production in pasture-fed ruminants. In addition, it was also to generate mechanistic information to understand the actions of insulin and supplemental protein infusion in the regulation of milk protein synthesis.

1.8 Thesis objectives

When the project on which this thesis is based was designed, the results of the studies by McGuire *et al.* (1995) and Griinari *et al.* (1997) had recently been published. The overall objective of this thesis was to determine the role of insulin in milk protein production in lactating pasture-fed ruminants. To do this, the HEC technique was used to examine if elevated concentrations of insulin, with or without protein supplementation increased milk protein yield and concentration as demonstrated by the studies in concentrate-fed cows.

To meet this overall objective, the first objective was to conduct an experiment in lactating ewes to set up the HEC technique for use in a subsequent experiment with lactating cows. These results were used to establish the response of the pasture-fed ewe to the HEC.

The second major objective was to conduct an experiment to determine the response of the lactating, pasture-fed dairy cow to the HEC. This experiment used the technique set up in first objective and allowed comparison of results with the lactating ewe study and overseas studies in concentrate-fed animals. Based on these, the third objective was determined.

The third major objective was to determine mammary gland utilisation of amino acids under the nutritional conditions imposed by the HEC. For this, the A-V concentration difference technique was used, in conjunction with a leucine tracer infusion. This was used to determine the utilisation of leucine in the mammary gland in lactating ewes and to determine if this was altered under HEC conditions.

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Chapter 2 General materials and methods.

Table 2-1 Locations for details of materials and methods used in this thesis.

Variable category	Variable	Location (section: *pg*)
Milk composition	Total nitrogen	3.3.3: *39*
	Non-protein nitrogen	3.3.3: *39*
	Crude protein	3.3.3: *39*
	Casein proteins	4.3.4.1: *64*
	Whey proteins	4.3.4.1: *64*
	Amino acids	5.3.3.1: *94*
	Fat content	3.3.3: *39*
	Fatty acids	4.3.4.1: *64*
	Lactose	3.3.3: *39*
	Minerals	4.3.4.1: *64*
Udder health	Somatic cell counts	4.3.4.1: *64*
Blood flow	Ultrasonic blood flow probes	5.3.3.4: *98*
	Fick principle	5.3.3.4: *98*
Blood composition	Glucose	3.3.3: *39*
	Insulin	3.3.3: *39*
	Insulin-like growth factor -I	4.3.4.2: *66*
	Non-esterified fatty acids	3.3.3: *39*
	β-hydroxy butyrate	3.3.3: *39*
	Triacylglycerols	4.3.4.2: *66*
	Plasma cortisol	4.3.4.2: *66*
	Amino acids	3.3.3: *39*
	Acetate	5.3.3.2: *95*
	Propionate	5.3.3.2: *95*
	Butyrate	5.3.3.2: *95*
Urine composition	Creatinine	5.3.3.2: *95*
	Cortisol	5.3.3.2: *95*
Feed	Dry matter intake	3.3.3: *39*
	Crude protein	3.3.3: *39*
	Energy	3.3.3: *39*

Chapter 3 The effect of insulin on the lactation performance of pasture-fed ewes

3.1 Abstract

Six lactating abomasally cannulated ewes were subjected to a hyperinsulinaemic euglycaemic clamp (HEC), with or without casein supplementation, in a two period cross-over design experiment. Casein supplementation by abomasal infusion significantly increased milk yield (1058 ± 40 vs 925 ± 40 g/d, $P<0.01$) and milk protein yield (64 ± 3 vs 51 ± 3 g/d, $P<0.01$). There was no effect of the HEC on milk protein output. There was a significant decrease in feed intake ($P<0.01$) during the HEC that contributed to a significant increase in the efficiency of dietary crude protein utilisation for milk protein production ($P<0.001$) in both the casein infused and control ewes. There was also a significant period effect across all measured parameters, which could be a stage of lactation effect due to the rapid decline of milk production in ewe lactation. In this study, in contrast to concentrate-fed cows, the use of the HEC with or without protein supplementation did not increase milk protein production in pasture-fed lactating ewes.

3.2 Introduction

The challenge that the international dairy industry faces is to increase milk protein production, either through an increase in milk protein yield or protein concentration. However, current understanding of dietary utilisation for milk production does not allow reliable management of dairy cows to consistently increase milk protein content. A more detailed understanding of mammary gland metabolism is needed to achieve this.

Early studies that examined the role of insulin in lactation showed that milk protein concentration but not yield could be increased (Schmidt 1966). However, these studies were confounded by the hypoglycaemic effect of insulin administration status (Kronfeld *et al.* 1963, Schmidt 1966). However, a recent study using the hyperinsulinaemic euglycaemic clamp technique (HEC) in concentrate-fed cows (McGuire *et al.* 1995) demonstrated that manipulating insulin status while maintaining euglycaemia through a simultaneous glucose infusion increased milk protein yield. Subsequent studies have

shown that when insulin and glucose status is manipulated via HEC in conjunction with supplemental dietary protein, milk protein yield is substantially increased (25-28%, Griinari *et al.* 1997a, Mackle *et al.* 1999). These results are remarkable when considered in relation to adaptations of whole body metabolism to meet the requirements for lactation, which seem to be aimed at blunting the effects of insulin, particularly in adipose and muscle tissue. However, not all HEC studies have shown an increase in milk protein production (Tessuraud *et al.* 1992, Annen *et al.* 1998). Therefore, it needs to be established if insulin regulates pathways responsible for controlling milk protein synthesis directly or indirectly (possibly through changes in IGF system as suggested by McGuire *et al.* 1995, Griinari *et al.* 1997a). Additionally, it should be questioned whether this increase in milk protein is a response to the constant increase in circulating insulin concentrations or is it an adaptation of these cows to the altered nutritional status induced by the HEC.

In New Zealand, the dairy industry has a commitment to increasing milk protein production in pasture-fed cows. However, ryegrass/white clover pastures are often high in crude protein and neutral detergent fibre and low in non-structural carbohydrate compared to diets recommended overseas (e.g. NRC) for lactating dairy cows (Carruthers *et al.* 1996). The milk protein response seen in the HEC studies has been in 'well fed' cows utilising a concentrate diet that is formulated to supply excess dietary requirements (Mackle *et al.* 2000). Consequently, there is a need to determine if the HEC will stimulate a milk protein response in pasture-fed cows that are fed a diet of low energy content.

The experiment reported in this chapter is the first in a series of 3 experiments designed to evaluate the response of lactating ruminants to the HEC. In this first experiment, pasture-fed lactating ewes were used to establish the methodology to allow a subsequent HEC experiment in lactating dairy cows.

There were a number of objectives that included:

- To establish the response of pasture-fed ewes to the HEC.
- To assess if lactating ewes are suitable models for lactating cows in future metabolic studies.

- To establish the HEC technique in pasture-fed ruminants i.e. conducting simultaneous insulin and glucose infusions while maintaining euglycaemia.
- To develop skills to work with surgically prepared animals as all ewes had an abomasal cannula implanted before lambing, so post-surgery care was required.
- To validate some of the analytical methods required for the HEC study, including the use of NIR for analysing ewe's milk and commercial blood glucose meters for rapid monitoring of blood glucose concentrations in ewes.

Preliminary data analysis on average milk yield has been published (Back *et al.* 1998).

3.3 Materials and methods

3.3.1 Animals

Six 3 and 4 year old Romney cross, lactating ewes with abomasal cannulae were housed indoors and individually fed (offered approximately 3 kg dry matter (DM)/day, sufficient to permit *ad libitum* intake) a diet of perennial ryegrass (*Lolium perenne*)-white clover (*Trifolium repens*) pasture during a 32 day experimental period. The pasture was cut daily and offered every 6 h, at 1200, 1800, 0000 and 0600 h, with water available *ad libitum.* The cut pasture was stored in a chiller at 4°C until fed.

The ewes were housed indoors for 14 days before surgery to acclimatise to being housed indoors. Feed was removed 12 hours before surgery but access to water was maintained at all times. The abomasal cannula were fitted at approximately 140 days gestation under general anaesthesia (3.5 ml saffon (10 mg/ml alphaxalone) and 30 ml breathable halothane (halothane B.P. 1 ml/ml)) using the method of Hecker (1974) by Dr G Renolds and P Back. Post surgery care included 5 mls penecillin (Bomacillin LA, propacain penecillin 150 mg/ml and benzathine penecillin 112.5 mg/ml, Vetpharm, New Zealand) being administered daily for 3 days to help prevent infection and rectal temperatures were also monitored. All ewes recovered from surgery with no signs of infection or any other progblems. Sutures were removed 7-10 days after surgery and the ewes were returned to pasture for lambing.

Three days after parturition the lambs were removed and the ewes were then housed indoors in metabolism crates for a 32 day experimental period. They were milked twice

daily throughout the trial period using a portable milking machine following an intravenous injection of oxytocin (1 IU/ewe, Oxytocin V, Vetpharm, NZ) to stimulate milk letdown. Two days before the start of infusions, 3 jugular catheters (1.2mm x 0.8 mm ID single lumen pvc tubing, Critchley Electrical Products Pty Ltd, Auburn NSW 2144, Australia), were implanted under local anaesthesia (Lopane, Lignocaine Hydrocholride U.S.P. 20 mg/ml; Ethical Agents Ltd, NZ) for infusions and blood sampling. Two catheters were implanted in the right jugular vein for glucose and insulin infusion and the remaining catheter in the left jugular vein for blood sampling.

To insert the jugular catheters, the ewes necks were clipped clear of wool in a patch 10 cm x 10 cm around the jugular veins. A 14 G catheter needle was used to access the vein and the pvc catheter fed 20 cm into the vein. The catheter needle was removed and the pvc catheter pulled back 5 cm and stitched into place with a suture next to the entry puncture. The catheter was further secured by fabric strapping (Elastoplast, Smith & Nephew, SA) glued (Ados) over the catheter (2 x 5 cm strips longwards and 2 x 5cm strips crosswards) on the shaved part of the neck. The catheter was then threaded through the wool to the back of the neck and tied with string elastic. To further protect the catheter, a collar of surgical elastic netting (Setonet, Seaton Healthcare Group PLC, Oldham, UK) was placed over top. All catheters were flushed with heparinised saline (100 IU/ml, New Zealand Pharmaceuticals, Palmerston North, New Zealand), filled and capped to keep patent between samplings. As two catheters were inserted in the right jugular vein, the bottom one put in first to prevent spearing it when the second one was put in. All catheters remained patent for the experimental period.

3.3.2 Experimental Procedure

Each ewe was randomly allocated to a treatment group in a crossover design where each animal was subjected to a HEC twice, with or without an abomasal infusion of casein. The experiment consisted of two 12 day periods, where (in each period) the first 4 days allowed acclimatisation to casein or control (water) infusions, with measurements being taken during days 5 to 8 to evaluate the effect of the casein infusion. All ewes were then subjected to a HEC from day 9 until day 12.

The casein (sodium caseinate, ICN Biomedicals Inc, Ohio.) solution (50 g casein/day in 780 mls of water) or water (780 ml/day) was infused directly into the abomasum of each ewe for 12 days. For the HEC (on days 9-12), bovine pancreas derived insulin (Sigma Chemicals, St Louis, MO.) was administered constantly (1 μg/kg $BW^{0.75}$ /h) in a sterile filtered 0.5% bovine serum albumin solution (Immuno Chemical Products Ltd, NZ). Once the insulin infusion was seen to be reducing circulating blood glucose concentrations, a glucose infusion was started at half glucose entry rate (as determined by Rutter & Manns 1985) to maintain euglycaemia. A sterile 25% w/w glucose solution (prepared using food-grade dextrose monohydrate (Pure Chem Co. Ltd, Thailand) and autoclaving for 20 min) was used to maintain euglycaemia via variable speed peristaltic pumps. Blood glucose concentrations were monitored using an Advantage Blood Glucose Meter (Boehringer Mannheim Ltd, NZ). This allowed rapid (within 2 min) determination of blood glucose concentrations for maintaining euglycaemia with the glucose infusion.

3.3.3 Sample collection and analyses

The ewes were milked twice daily at 0800 and 2000 h, and the yields weighed and a sample stored at -20°C for further analysis. For analysis, the samples were thawed at room temperature. They were then warmed in a water bath (<37°C so that proteins were not denatured) and mixed by gentle agitation. Milk composition was measured by near infrared spectroscopy in transmission mode (NITS, model 6500, NIRsystems Inc, Silver Spring, MD, USA) with PC software by Infrasoft International (version 3.1). The NITS was calibrated for ewe's milk using the following analyses: Crude protein was calculated as Total N x 6.38, with Total N being determined by combustion on a Carlo Erba NA1500 Nitrogen analyser. Non-protein nitrogen (NPN) was determined using IDF standard 20B modified for determining ammonia by flow injection analysis. True protein was then calculated as Total N - NPN x 6.38. Fat content was determined gravimetrically following the extraction of the fat by diethyl ether (modified AOAC method 963.15, AOAC 1990). Lactose concentration was determined by HPLC, using a Shimadzu LC4A system with an anion-cation exchange resin column (Aminex HPX-87P) at a temperature of 60°C with a water mobile phase and a flow rate of 0.6 ml/min. Lactose was detected using a Waters refractive index detector (for full method see Appendix A).

The ewes were fed 4 times daily and the amount of feed offered was recorded. Refusals from each feed were collected from the bins and floor, bulked separately, weighed and the bin refusals subsampled for analysis. Dry matter was calculated on both feed offered and refused and daily dry matter intake (DMI) was measured as that offered less that refused. Daily samples were analysed for crude protein (CP) and energy (ME) by near infrared spectroscopy in reflectance mode (NIRS) calibrated for mixed pasture (Corson *et al.* 1999).

During days 5-9, blood samples were taken at 1200, 1400 and 1600 to establish basal concentrations of glucose, insulin, non-esterified fatty acids (NEFA), β-hydroxybutyrate and amino acids (AA). An average glucose concentration was determined, which was the target euglycaemia (± 10%) for each individual animal during the HEC. To assess the effect of the HEC on circulating concentrations of insulin, glucose, NEFA, AA and β-hydroxy butyrate, blood samples were taken over the 4 days of the HEC. During the first 24 h, blood samples were taken at 1400, 1600, 1800, 0000, and 0600. Thereafter, on days 2, 3 and 4 of the HEC, samples were taken at 1200, 1400 and 1600.

All blood samples were collected with disodium ethylenediaminetetraacetate (Na_2.EDTA) as the anticoagulant. A subsample (1ml) of whole blood was deproteinised by addition of 0.5 ml 30% trichloroacetic acid (TCA), centrifuged at 4°C for 20 min and filtered through a 0.45 μm cellulose syringe filter. This filtrate was used to measure circulating AA concentrations by two methods: Amino acid concentrations (exclusive of cysteine) were determined by reverse phase HPLC separation of phenylisothiocyanate derivatives on a Waters PicoTag© column used in conjunction with a Shimadzu LC10/A HPLC system using a method modified from Bidlingmeyer *et al.* 1984 (for full method see Appendix B). Cysteine concentrations were determined by a modified automated method using acid ninhydrin (Gaitonde 1967). Concentrations of tryptophan are not reported because of the uncertainty in the proportion of tryptophan associated with plasma protein that is released by the extraction method. The remaining blood was centrifuged (at 3270 g at 4°C for 15 min) and the resulting plasma was harvested and stored at -20°C until analysed. Amino acid concentrations are grouped as essential (EAA) or non-essential (NEAA) as defined for lactating dairy cows by Clark

et al. (1977), which has been validated for lactating ewes by Fleet & Mepham (1985). On this basis the EAA include arginine, cysteine, histidine, leucine, lysine, isoleucine, methionine, phenylalanine, threonine, tyrosine and valine.

Plasma insulin concentrations were measured using a double antibody radioimmunoassay (Flux *et al.,* 1984). Intra- and inter-assay coefficients of variation were 8.7% and 12.9% respectively. The mean sensitivity was 22.7 pg insulin/ml. Plasma NEFA and β-hydroxy butyrate concentrations were measured by enzymatic colorimetric methods that have been adapted to a Cobas Fara II autoanalyser (Hoffman-La Roche Ltd, Basal, Switzerland). The NEFA method is based on that of McCutcheon & Bauman (1986), utilising a Wako C test kit (Wako Pure Chemical Industries Ltd, Osaka, Japan) and the intra- and inter- assay coefficients of variation were 4.6% and 2.3% respectively. The β-hydroxy butyrate method is based on that of Williamson & Mellanby (1974). The intra- and inter- assay coefficients of variation were 4.7% and 2.1% respectively.

3.3.4 Statistical analysis

As previously stated, the experiment was conducted with a two period cross-over design, with 6 ewes randomly assigned to two groups. The data generated were analysed with a split plot analysis with repeated measures. The casein treatment was considered as the main plot, with the HEC as the split plot and time was used as the repeated factor. Analyses were performed using the procedure GLM from the statistical package SAS (1988). The model used for the analyses was:

$$Y_{ijkl} = \mu + \delta_k + \pi_{(l)k} + \alpha_i + \beta_j + \varepsilon_{ijkl}$$

where μ is the mean, δ_k is the fixed effect due to the group k, $\pi_{(l)k}$ is the l^{th} ewe in group k, α_i is the fixed effect due to the treatment i, β_j is the fixed effect due to period j, and ε_{ijkl} is the error term. Results are expressed as least squares means ± standard errors. All treatment and period effects and their interactions were tested. Where there was a significant effect of treatment, probability values were generated to compare between treatments. Normality of the data was tested by plotting the standardised residuals against the standardised predicted values of the response variables. No plots showed a pattern that would indicate that the normality assumption of the ANOVA model should be questioned and that the data should be transformed.

3.4 Results

Abomasal infusion of casein had no effect on DMI and subsequently no effect on diet CP or ME intakes as pasture quality did not vary greatly. However, the HEC caused a decrease in DMI, and accordingly decreased CP and ME intake in both the casein infused and control groups (Table 3-1). There was a significant effect of period of infusion on DMI ($P<0.01$) as a result of a larger reduction of intake during the second period of insulin infusion, particularly in the control group (Fig. 3-1). Dietary CP intake ($P<0.05$) was also significantly influenced by period of infusion but ME intake was not. The data were also tested to see if there was a sequence effect of casein infusion i.e. if there was a difference in effect when the ewes received casein supplementation during the first or second period of infusion. There was a significant sequence effect on DMI ($P<0.01$), dietary CP ($P<0.001$) and ME ($P<0.01$) intakes.

To account for the additional crude protein or energy supplied by the casein or glucose infusions, the amount supplied by the infusions was added to the dietary CP and ME intakes (Total ME and Total CP in Table 3-1). The Total CP and ME intakes were not significantly different during the casein infusion. However, by the fourth day of the HEC, Total CP and ME intakes of the control group were significantly lower than the casein infused group. While there was a significant period of infusion effect on Total CP ($P<0.01$), there was no effect on Total ME. There was however, a significant sequence effect on both Total CP ($P<0.01$) and Total ME ($P<0.01$).

Energy balance of the ewes was not influenced by casein treatment, period of infusion or sequence of infusion (see Table 3-1). The HEC caused the negative energy balance of the casein infused ewes to decrease slightly, whereas there was no significant change in the control ewes. The efficiency of CP utilisation (a measure of the gross efficiency of dietary CP utilisation for milk protein production) was significantly higher in the casein infused ewes during both the casein infusion and HEC. However, efficiency of utilisation increased significantly in both groups during the HEC (Table 3-1).

Table 3-1 Comparison of feed intakes and energy balance (LSMeans ± SEM) on day 4 of the casein infusion and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC		
	Casein	Control	Casein	Control	± SEM
Intakes					
DMI (kg/d)	1.06[a]	1.06[a]	1.00[a]	0.85[b]	0.04
Dietary CP (g/d)	200.0[a]	220.0[a]	163.0[b]	133.0[c]	10.0
Total CP (g/d)[1]	250.0[a]	220.0[a]	216.0[a]	133.0[b]	16.0
Dietary ME (MJ ME/d)	12.0[a]	13.1[a]	10.5[b]	9.1[c]	0.4
Total ME (MJ ME/d)[2]	12.8[a]	13.1[a]	14.3[b]	12.1[a]	0.4
Energy					
Energy balance[3]	-16.8[a]	-15.7[a]	-14.2[b]	-15.7[a]	0.5
Efficiency of CP utilisation (g/g)[4]	0.32[a]	0.24[b]	0.45[c]	0.36[a]	0.02

Treatments: Infusion = casein vs buffer infusion, HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused group, control = water infused group.
[a,b,c] LSMeans with different superscripts are significantly different at P<0.05.
[1,2] Total energy (MJ ME) and crude protein (CP) intakes were calculated by combining dietary intake with additional ME or CP available from the glucose and casein infusions.
[3] Calculated as total energy intake (MJME) from diet + casein + glucose – energy output (MJME) estimates of maintenance requirements + the energy content of milk produced (4.7 MJME/kg).
[4] Calculated as g milk protein produced per g of CP intake.

Abomasal infusion of casein supported greater milk yield, crude protein, total protein, lactose and solids yield than the control infusion of water. When both treatments were subjected to the HEC, this did not significantly alter yield of milk or milk components in either the casein infused or control ewes. Neither the casein infusion nor HEC had any effect on the concentration of milk solids, CP, or lactose (Table 3-2). In contrast, milk fat yield and concentration steadily decreased during the HEC. There was a significant effect of period of infusion on milk yield ($P<0.01$), and all milk component yields (milk CP $P<0.01$, fat $P<0.01$, lactose $P<0.05$, solids $P<0.05$). There was also a significant period of infusion effect on milk fat ($P<0.001$) and solids ($P<0.001$) concentrations but not lactose and crude protein concentrations.

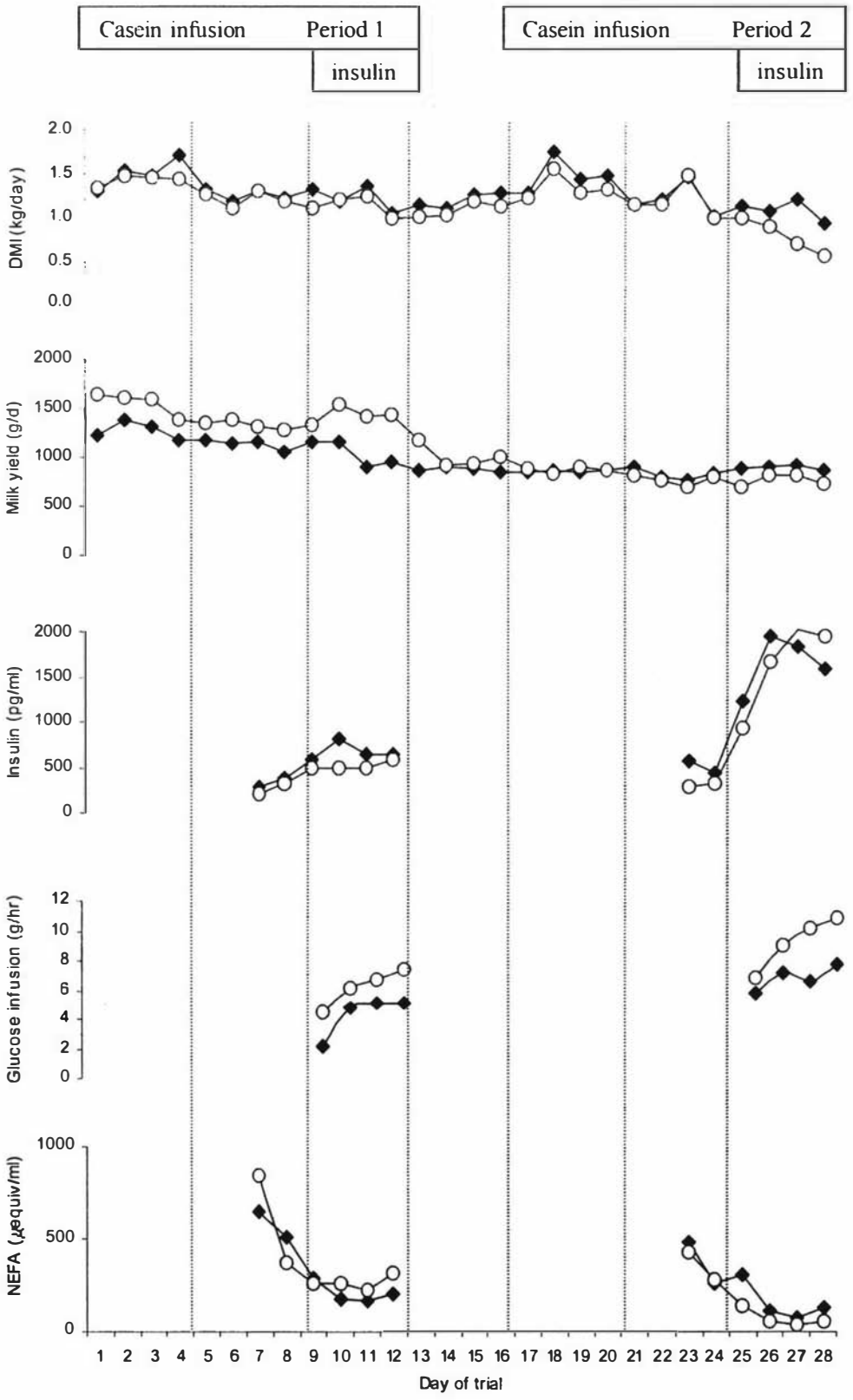

Fig. 3-1 Plots of group means generated from raw data to illustrate the type of changes that occurred over 2 periods of insulin infusion during the 28 day experiment. No statistical inferences have been drawn, so no error bars are plotted. Group 1 (O) received supplementation during the first period of infusion. Group 2 (◆) received casein supplementation during the second period of infusion.

Table 3-2 Comparison of milk yield and milk component yields (LSMeans ± SEM) on day 4 of the casein infusion and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC		
	Casein	Control	Casein	Control	± SEM
Milk yield (g/d)	1058[a]	925[b]	1141[a]	836[b]	40
Component yield (g/d)					
Total milk solids[1]	210[a]	179[b]	202[a]	142[b]	12
Crude protein	64[a]	51[b]	68[a]	46[b]	3
Fat	80[a]	72[ab]	63[b]	46[c]	5
Lactose	49[a]	41[b]	53[a]	36[b]	2
Concentration (%)					
Milk solids	19.4[a]	19.7[a]	17.4[b]	17.4[b]	0.4
Crude protein	6.1	5.8	6.0	5.8	0.2
Fat	7.4[a]	7.8[a]	5.5[b]	5.6[b]	0.3
Lactose	4.5	4.6	4.5	4.5	0.1

Treatments: Infusion = casein vs buffer infusion, HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused group, control = water infused group.
[a, b, c] LSMeans with different superscripts are significantly different at P<0.05.
[1]Milk solids = weight of total milk solids as determined by freeze drying.

There was an effect of the order of receiving casein supplementation on the yield of total milk solids (P<0.05), crude protein (P<0.01) and lactose (P=0.08) (data not shown).

Circulating concentrations of AA were measured on day 4 of the casein infusion, during the first 24 h of the HEC, and on day 4 of the HEC. Casein infused ewes generally had higher circulating concentrations of EAA and NEAA than control ewes (Table 3-3). There was no significant period of infusion or sequence effects on circulating AA concentrations.

Circulating concentrations of AA were compared between day 4 of the casein infusion, after the first 24 h and on day 4 of the HEC. Circulating concentrations of most EAA (except for histidine and tyrosine, Fig. 3-2a), significantly decreased over the four days. However, the effect of the HEC on individual AA appeared to vary in that the severity

and rate of decline differed between AA. Circulating concentrations of BCAA (Fig. 3-2b), threonine and arginine decreased dramatically over the first 24 h of insulin infusion in casein infused and control ewes. Concentrations of these AA in the casein infused ewes were a similar concentration on day 4 to those measured at 24 h, whereas concentrations in the control ewes continued to decline after the first 24 h and were significantly lower by day 4 of the HEC (Fig. 3-2).

Table 3-3 Comparison of concentrations of circulating amino acid in plasma (LSMeans ± SEM) on day 4 of the casein infusion and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC		
	Casein	Control	Casein	Control	± SEM
Essential amino acids (μM)					
Arginine	157.9^{a}	114.5^{b}	88.8^{c}	59.2^{d}	7.5
Cysteine	64.9^{a}	57.3^{b}	55.8^{b}	44.5^{c}	2.4
Histidine	38.9^{ab}	35.5^{b}	42.6^{a}	35.4^{b}	2.3
Leucine	128.8^{a}	93.5^{b}	81.6^{b}	56.6^{c}	5.4
Lysine	161.5^{a}	106.7^{b}	123.1^{ab}	85.4^{b}	13.2
Isoleucine	103.6^{a}	81.7^{b}	60.9^{c}	42.5^{d}	5.2
Methionine	27.7^{a}	17.7^{b}	20.7^{b}	16.3^{b}	1.6
Phenylalanine	53.7^{a}	45.9^{ab}	47.2^{b}	41.5^{b}	2.5
Threonine	110.9^{a}	90.3^{ab}	81.2^{b}	50.8^{c}	7.1
Tyrosine	73.0	58.0	64.9	47.5	10.5
Valine	207.7^{a}	148.7^{b}	126.9^{b}	80.3^{c}	11.2
Nonessential amino acids (μM)					
Alanine	112.7^{a}	95.2^{a}	98.2^{a}	76.6^{b}	6.2
Glycine	450.1^{a}	516.6^{ab}	541.5^{b}	577.2^{b}	30.8
Ornithine	68.9^{a}	49.5^{b}	38.4^{c}	22.0^{d}	3.6
Proline	124.1^{a}	81.8^{b}	104.5^{a}	61.5^{c}	7.2
Serine	90.1	87.7	84.2	76.3	4.7

Treatments: Infusion = casein vs water infusion, HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused group, control = water infused group.
$^{a, b, c, d}$ LSMeans with different superscripts are significantly different at $P<0.05$.

Lysine, phenylalanine (Fig. 3-2c) and methionine concentrations also fell significantly during the first 24 h of the HEC in the casein infused ewes and were a similar concentration on day 4 to those measured at 24 h. Although concentrations of these three AA decreased during the HEC in the control ewes, these reductions were not statistically significant. Cysteine concentrations decreased significantly over the first 24

h of the HEC and were a similar concentration on day 4 in the casein infused ewes. In comparison, concentrations of cysteine in the control ewes tended to decrease (P=0.06) over the first 24 h of the HEC, but were significantly lower by day 4 relative to pre-HEC concentrations.

Of the NEAA that were determined, serine, proline and alanine concentrations significantly decreased over the first 24 h of the HEC and were measured at a similar concentration on day 4 in the casein infused group. In the control ewes, the rate of decline in circulating concentrations of proline and alanine was much slower but significant on day 4. There was no significant change in serine concentrations in the control ewes, whereas concentrations of ornithine decreased dramatically in both groups over the first 24 h of the HEC and had not fallen further on day 4. Plasma concentrations of glycine increased in both groups during the HEC, although the increase was only significant in the ewes infused with casein.

Abomasal infusion of casein had no effect on blood glucose or plasma insulin during the casein infusion period or the HEC (Table 3-4). It also had no effect on the amount of exogenous glucose required to maintain euglycaemia, although glucose required to maintain euglycaemia increased significantly over the four days of the HEC in both casein infused and control ewes at a similar rate (5.4 ± 0.4 to 7.8 ± 0.3 g/h, $P<0.001$). The amount of glucose required per h was also significantly different between the two periods of insulin infusion. During the first period of insulin infusion, glucose requirements increased from 4.5 ± 0.5 to 6.3 ± 0.5 g/h ($P<0.05$). In comparison, glucose requirements during the second period of insulin infusion increased from 6.3 ± 0.5 to 9.3 ± 0.5 g/h ($P<0.001$, Fig. 3-1).

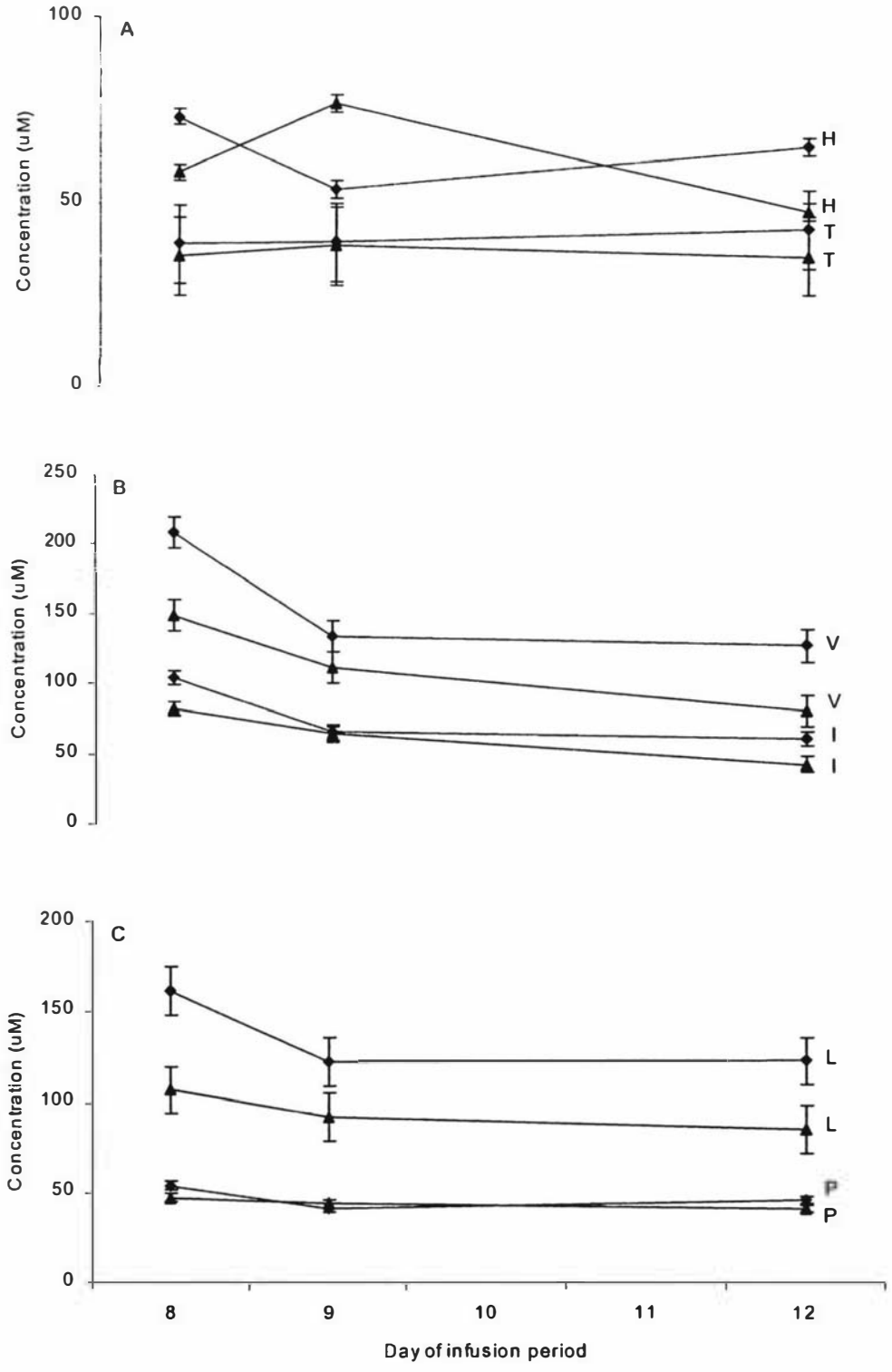

Fig. 3-2 The response of selected essential amino acids to the hyperinsulinaemic euglycaemic clamp (HEC), where concentrations were measured on day 8 of infusion period (day 4 casein infusion), day 9 (day 1 HEC) and day 12 (day 4 HEC). Treatments are shown by: casein infused ewes (◆) and control ewes (▲). Amino acids were: histidine (H), tyrosine (T), valine (V), isoleucine (I), lysine (L) and phenylalanine (P).

The HEC increased plasma insulin concentrations more than threefold above basal values in both casein infused and control groups (Table 3-4). Despite the insulin infusion rate being held constant between the two infusion periods, the increase in circulating concentrations was significantly greater during the second period of insulin infusion ($P<0.001$, see Fig. 3-1). The mean blood glucose concentration for euglycaemia was maintained during the insulin infusion (see Table 3-1), although average concentrations differed slightly between the two infusion periods (3.0 ± 0.1 vs 3.3 ± 0.1 mM).

Table 3-4 Comparison of circulating concentrations of insulin and energy metabolites (LSMeans ± SEM) on day 4 of the casein infusion and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC		
	Casein	Control	Casein	Control	± SEM
Insulin (pg/ml)	365[a]	297[a]	985[b]	1385[b]	130
Blood glucose (mM)	3.2	3.1	3.0	3.2	0.2
NEFA (μequiv/ml)	312[a]	395[a]	224[b]	131[c]	30
β-hydroxybutyrate (mM)	0.4[a]	0.39[a]	0.22[b]	0.17[b]	0.02

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused group, control = water infused group.
LSMeans with different superscripts are significantly different at $P<0.05$.

Circulating concentrations of plasma NEFA tended to be higher in control ewes during the casein infusion whereas there was no difference between groups in β-hydroxybutyrate concentrations (Table 3-4). During the four days of the HEC, concentrations of plasma NEFA and β-hydroxybutyrate decreased significantly in both groups, and were significantly lower during the second period of insulin infusion ($P<0.05$, $P<0.05$ respectively).

3.5 Discussion

The experiment reported in this chapter is the first in a series of 3 experiments designed to evaluate the response of lactating ruminants to the HEC to determine the role of

insulin in milk protein synthesis. In this first experiment, the aims of this study were to set up the HEC technique and then examine if, under HEC conditions, milk protein synthesis was altered in pasture fed lactating ewes. When reviewing the results from this experiment and comparing it to studies in concentrate fed cows and goats, it should be recognised that a) the ewes were from a commercial flock where the lactation lengths are short (generally around 12 weeks, with a peak yield 2-4 weeks after lambing, (Rattray 1986) and b) they have different AA requirements to dairy cows in that wool growth has a particular requirement for lysine and the sulphur AA (cysteine and methionine) (reviewed by Reis and Sahlu 1994).

Use of the HEC, with or without supplemental protein, did not alter milk protein production in this study with pasture-fed ewes. This is in contrast to experiments with concentrate-fed cows (McGuire *et al.* 1995, Griinari *et al.* 1997a, Mackle *et al.* 1999, 2000), and goats (Bequette *et al.* 2001) where milk protein production was increased. However, in this study milk protein yield was maintained despite a large decrease in dietary crude protein intake. The changes in gross efficiency of dietary crude protein utilisation indicate that nutrient partitioning to the mammary gland was changed to support milk protein output. This is in contrast to milk fat yield, which decreased during the HEC. There were clearly different responses to the casein and insulin infusions, and statistical analysis shows the data generated from this experiment were influenced by period of insulin infusion, stage of lactation and sequence of casein infusion. Although the experimental design was the same as the HEC studies with concentrate-fed cows and goats, these factors may have contributed to the different response in the pasture-fed ewes.

The response to casein infusion shows the ewes in this study utilised the abomasal casein infusion to produce greater milk and hence milk protein yields than the control group. This is consistent with studies in pasture-fed ewes (Barry 1980) and cows (Rogers & McLeay 1977), and concentrate-fed cows (Clark *et al.* 1977, Griinari *et al.* 1997a). Milk, milk protein yield and concentration were not altered in either the casein supplemented or control group during the HEC despite the reduction in feed intake. That insulin infusion had no effect on milk yield has been demonstrated in both short (2.5-10 h, Hove 1978, Laarveld *et al.* 1981, Tesseraud *et al.* 1992) and longer (4 d) studies (McGuire *et al.* 1995, Griinari *et al.* 1997a). However, milk and milk protein

yield was significantly greater in both groups during the first period of insulin infusion and the magnitude of difference in yield between the two groups was also greater. This resulted in a significant sequence effect, which indicates that the sequence that the ewes received the casein infusion may have influenced the maintenance of milk yield throughout the study, as the ewes who received the casein infusion first produced more milk over the whole experimental period.

The changes in circulating concentrations of AA during the HEC in this study are similar to those reported in other studies of this type with cows (McGuire *et al.* 1995, Griinari *et al.* 1997a, Mackle *et al.* 1999) and goats (Tesseraud *et al.* 1992, Bequette *et al.* 2001). Several mechanisms may have caused this reduction. The HEC, with or without AA infusion, has been shown to increase hind-limb blood flow and AA uptake in lactating goats (Bequette *et al.* 2001). However, although AA uptake was increased, it is unclear how the HEC changes AA use in terms of protein synthesis and degradation. In lactating goats under HEC conditions, Tesseraud *et al.* (1993) reported no increase in muscle protein synthesis and a decrease in whole body protein degradation. Tauveron *et al.* (1994) also found no increase in muscle protein synthesis. An increase in AA uptake by the mammary gland would also contribute to the decrease in circulating concentrations of AA. The large decrease seen in BCAA and arginine may occur because of increased use as a nitrogen supply to synthesise NEAA in the mammary gland. Alternatively, insulin may reduce AA release because of decreased protein degradation in splanchnic tissue (reviewed by Biolo & Wolfe 1993, Grizard *et al.* 1995).

In this study, maintenance of milk protein output despite the reduction in feed intake may have been a result of increased uptake of AA by the mammary gland due to the effect of insulin on AA transport systems. Several studies (reviewed by Baumrucker 1985, Guidotti *et al.* 1978) indicate that insulin has the potential to increase carrier protein synthesis and maximise transport velocity of AA. Insulin stimulates the A transport system, which can transport most AA (Le Cam & Freychet 1978) and insulin may also prevent degradation and inactivation of the carrier proteins (Elias *et al.* 1971). The slower rate of decline in circulating AA in control ewes may be due to a longer time lag for synthesising new carrier proteins. Other possible mechanisms that contributed to maintaining milk protein production are that a) insulin stimulated an

increase in number of productive secretory cells in the mammary gland. Molenaar *et al.* (1992) reported that in bovine and ovine mammary tissue not all cells were actively lactating, indicating that there exists the potential for the mammary gland to increase milk protein synthesis if more cells are 'switched on'. b) insulin stimulates an increase in mammary blood flow, thereby increasing the supply of precursors to the gland. Increased mammary blood flow in response to the HEC (alone and with supplemental protein/AA) has been reported in cows (Mackle *et al.* 2000) and goats (Bequette *et al.* 2001).

Milk fat yield and concentration were significantly decreased during the HEC in both casein infused and control ewes. This is in contrast to McGuire *et al.* (1995) and Griinari *et al.* (1997b) where there was no reduction in milk fat (although in the later study there was a slight reduction in percentage). In the study by Mackle *et al.* (1999) milk fat concentration was reduced by the insulin infusion but there was a reduction in milk fat yield only in the water infused group. Insulin has no direct effect on uptake of milk fat precursors by the mammary gland (Laarveld *et al.* 1985), or the rate of milk fat synthesis (Laarveld *et al.* 1985, McGuire et at 1995, Griinari *et al.* 1997b). However, insulin can influence the supply of milk fat precursors such as fatty acids and β-hydroxybutyrate to the mammary gland by altering hepatic and adipose tissue metabolism. Insulin reduces NEFA release from adipose tissue and ketone body production and gluconeogenesis (Brockman 1990). In pasture-fed ewes, milk fat content was positively related to body fat mobilisation (Geenty & Sykes 1986). In this study, circulating concentrations of NEFA and β-hydroxybutyrate were significantly reduced during the insulin infusion (Table 3-4), indicating reduced adipose tissue mobilisation and hepatic ketone body production. This has implications for supply of milk fat precursors and energy substrate to the mammary gland. The decreased availability of NEFA and β-hydroxybutyrate (and possibly acetate) would contribute to reduced milk fat synthesis but no samples were analysed to determine if there was a change in fatty acid composition.

Energy balance is one of the major differences between this study in pasture-fed ewes and the studies in concentrate-fed cows and it may be energy balance that potentially limited any milk protein response in these pasture-fed ewes. In this study, the ewes

were calculated to be in negative energy balance and losing weight throughout the study, whereas the cows were reported to always be in positive energy balance. Energy balance values were calculated from Geenty & Sykes (1986) for lactating ewes at pasture. Although liveweight decreased in all ewes over the experimental period, no statistical analysis was performed due to the confounding effect of reduced intake on gut fill, as the effect of the HEC on intake was different between periods of insulin infusion. However, lactating ewes of similar liveweights to those in this study have daily ME requirements of 25-30 MJ ME/day and need to consume between 2.2-2.6 kg DM/day to meet requirements. Even adjusting for the casein and glucose infusions (Table 3-1) the ewes consumed consistently less than requirements and hence were always in negative energy balance. This is consistent with work of Geenty & Sykes (1986) who reported that pasture-fed ewes, irrespective of herbage allowance and milk production (machine milked or suckled) were in negative energy balance during the first 6 weeks of lactation.

The demand for exogenous glucose increased similarly over the 4 days of the HEC in casein infused and control ewes, with a significantly greater demand for exogenous glucose in the second period of insulin infusion. The increase in demand for exogenous glucose was accompanied by a gradual decrease in DMI, particularly during the second period of insulin infusion. This decrease in intake and increased reliance on exogenous glucose has also been shown in other HEC studies (McGuire *et al.* 1995, Griinari *et al.* 1997a, Annen *et al.* 1998). It would appear that the ewes matched intake and glucose infusion, so that on a ME basis, there was no difference between casein or insulin infusions, and they did this by reducing feed intake and relying on exogenous glucose. However, in the study of Griinari *et al.* (1997a), energy balance in the casein infused group was more than doubled, which potentially was available for milk protein synthesis, together with the supplemental protein (casein). Ulyatt (1997) suggests that production responses to supplemental protein in pasture-fed animals can occur if an animal is limited in protein and/or AA supply, or if there is surplus energy available at the tissue level. Therefore, in our study, although metabolism was changed sufficiently to maintain AA supply, there may have not been enough energy available to increase milk protein synthesis.

The ability of these ewes to respond to the insulin infusion may have also been influenced by difference in the stage of lactation from the first to the second period of infusion and by the changes in metabolic state of the ewe, especially the mammary gland, induced by the first period of insulin infusion. Despite keeping the insulin infusion rate constant, the increase in circulating concentrations of insulin, and the demand for exogenous glucose to maintain euglycaemia were significantly different between infusion periods. Both were significantly greater in the second period of insulin infusion. This may have been due to a change in tissue sensitivity to insulin so that insulin was not removed as quickly from the peripheral circulation. The rate of insulin clearance or rate of glucose uptake by the lactating ewe depends on the sensitivity of tissues to insulin (Vernon *et al.* 1990) and this changes over lactation. Basal plasma insulin concentrations have been shown to be lower in early lactation compared to mid lactation in goats (Debras *et al.* 1989) and dairy cows (Jenny *et al.* 1974) and insulin clearance (rate of removal) has been shown to be higher in lactating ewes than non-lactating ewes (Faulkner & Pollock 1990). In early lactation, low circulating concentrations of insulin allows higher mobilisation of adipose tissue to meet energy demands. However, in mid-lactation (or later) when milk production is not as high and the demand for energy reduced, circulating concentrations of both insulin and glucose increase (Jenny *et al.* 1974). The changes between the two infusion periods question the effects that the constantly high concentration of insulin has on tissue sensitivity and receptors. The data are consistent with the hypothesis that the first infusion pushed the ewes prematurely into a mid-late lactation condition. However, no work on tissue receptors or sensitivity has been done to elucidate this.

The changing concentrations of insulin and increasing consumption of glucose may also have contributed to the decrease in feed intake observed. The reduction in feed intake observed in this study is similar to decreases observed in other HEC studies (McGuire *et al.* 1995, Griinari *et al.* 1997a, Annen *et al.* 1998). Studies on factors controlling feed intake have demonstrated that an increase in circulating insulin concentrations and an adequate level of glucose has the potential to activate satiety centres in lactating and non-lactating sheep and depress feed intake (Deetz & Wangsness 1980, 1981, Rutter & Manns 1985). However, insulin may promote satiety only when sufficient energy metabolites are available (Deetz & Wangsness 1980, 1981). In our experiment, the daily requirement for exogenous glucose increased similarly in casein infused and control

ewes during the insulin infusion, potentially meeting the requirements of peripheral tissues and activating satiety centres in the brain. A significant period of infusion effect indicates there was a greater satiating effect during the second period of insulin infusion when there was a larger decrease in intake due to higher insulin concentrations and glucose use.

It is not clear how milk protein yield was maintained during the insulin infusion despite the estimated intake of dietary crude protein decreasing by up to 40 %. While the changes in circulating amino acids, NEFA and glucose infusion rate are similar to those reported in the HEC studies in concentrate fed cows, the significant period of infusion effects raises questions as to the use of a cross-over design for this type of experiment. These effects may have several causes: using lactating ewes which have a different lactation curve to dairy cows, or it may be due to the effect of the constant insulin infusion on tissue receptors and sensitivity.

3.6 Conclusion

In this study, it is possible that insulin affected both mammary gland and extra-mammary tissues to maintain milk protein synthesis by a) altering whole body metabolism, so that a 'sparing' effect on amino acids occurred, which maintained the supply of milk protein precursors to the mammary gland despite the reduction in feed intake, and b) directly influencing amino acid uptake by the mammary gland. This response to the HEC demonstrates the adaptive ability of lactating ewes to co-ordinate responses to major disturbances in metabolism, by concurrent or simultaneous changes in hepatic, muscle and adipose tissues to maintain metabolic balance across the whole body. However, it is not clear what drives these adaptive responses. There are many possible reasons but two that should be considered for future work are that the mammary gland detects changing AA concentrations at either the gut or gland and responds by regulating supply to the secretory cell. The signals that are involved and how they activate the secretory cell remains to be determined.

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Chapter 4 The effects of insulin on the production of pasture-fed Jersey cows

4.1 Abstract

Five lactating rumen fistulated Jersey cows were subjected to a hyperinsulinaemic euglycaemic clamp (HEC) with and without an abomasal infusion of casein in a two period cross-over design experiment. The casein infusion did not alter milk yield in supplemented cows (14.30 ± 0.45 kg/d) compared to the control cows (14.3 ± 0.5 kg/d). The HEC significantly reduced milk yield in the control cows compared to supplemented cows (9.9 ± 0.5 vs 13.2 ± 0.5 kg/d). Milk protein yield was significantly higher in the casein supplemented cows compared to the control cows (482 ± 16 vs 421 ± 16 g/d). The HEC significantly reduced milk protein yield in the control cows compared to supplemented cows (311 ± 16 vs 459 ± 16 g/d). Feed intake was significantly reduced in both the casein supplemented and control cows by the HEC. In contrast to results in concentrate fed cows, the use of the HEC technique did not alter milk protein yield in casein supplemented cows and reduced yield in control cows.

4.2 Introduction

This chapter reports the second of the three experiments that were designed to investigate the role of insulin in milk protein production in pasture-fed ruminants by manipulating the insulin and glucose status of the animals using the hyperinsulinaemic euglycaemic clamp (HEC) technique. In Chapter 3, use of the HEC technique in pasture-fed lactating ewes demonstrated that milk protein output was maintained despite a decrease in dry matter (DMI) and dietary crude protein intake. This indicated a change in nutrient partitioning, as shown by the change in the proportional utilisation of dietary protein for milk protein produced. However, that there was no milk protein response is in contrast to experiments where use of the HEC technique with and without supplementary protein, has resulted in increased milk protein output in concentrate-fed cows (McGuire *et al.* 1995a; Griinari *et al.* 1997a; Mackle *et al.* 1999, 2000) and goats (Bequette *et al.* 2001).

The results of the previous chapter indicate that the lactating ewe has the ability to respond to the HEC. However, it is not clear if the different response compared to concentrate fed cows or goats was because of species difference, energy balance or type of feeding.

Therefore, a second experiment was conducted to establish the response of pasture-fed cows to the HEC by applying the technique used in Chapter 3 to lactating Jersey cows fed pasture. This involved:

- Preparing the cows for the experiment, which included rumen fistulation and the adaptation and development of the casein infusion line for infusion into the abomasum. The cows were from a commercial herd and needed to be acclimatised to indoor housing and the infusion procedures.
- Adapting the HEC procedure and other methods developed in the lactating ewe trial (chapter 3) for use in pasture-fed dairy cows.
- Validating analytical methods and establishing a NIR calibration for cow's milk using standard analytical techniques.
- Establishing the response of lactating, pasture-fed dairy cows to the HEC and comparing this to the data from the ewe trial.

The main experiment reported in this chapter was a cross-over trial that examined the response of pasture-fed dairy cows to the HEC. In addition, a supplementary experiment was run concurrently which examined if chromium supplementation in times of stress such as early lactation improved production by improving glucose utilisation. Results from the chromium trial have been published elsewhere (Back *et al.* 1999). However, it is acknowledged here as one cow was used in both studies (section 4.3.5).

4.3 Materials and methods

4.3.1 Animals

Five lactating Jersey cows (approx 46 ± 14 d post partum, 352 ± 20 kg, (mean ± SD)) with rumen fistulae were housed indoors and individually offered sufficient pasture to permit *ad libitum* intakes of a diet of perennial ryegrass (*Lolium perenne*) -white clover (*Trifolium repens*) pasture during a 6 week experimental period from September -

November 1997. The pasture was cut twice daily and offered every six h at 0830, 1430, 2030 and 0230 h, with water available *ad libitum.*

The in-calf cows were rumen fistulated in May 1997 using the following method (as described by G. Waghorm, pers.comm). Briefly, the cows were sedated (0.85 ml Rompun® 2%, Bayer New Zealand Ltd, Auckland, New Zealand) and anaethatised with a L block using local aneasthesia (150 ml xylocaine, Ethical Agents, Auckland, New Zealand). A 13-15 cm verticle incision was made in the position of the leg of the inverted L. Blunt dissection was used to part the muscle layers to locate the rumen. The muscle layers and skin were sutured to the rumen and then an incision was made to insert a small 3 inch cannula. This was removed 12-14 days later and replaced with a larger 4 inch cannula. The cows received 20 ml antibiotic (Bomacillin LA®, procaine penicillin 150 mg/ml and benzathine penicillin 112.5 mg/ml), daily post fistulation to prevent infection.

The cows were milked throughout the trial period with a portable milking plant (Nu Pulse Porta Milker, Hamilton, NZ). Two days prior to the start of each experimental period, 2 jugular catheters (PVC single lumen tubing, ID 1.0.mm: OD 1.5 mm) were inserted into each of the jugular veins under xylazine hydrochloride sedation (0.7 ml Rompun® 2%, Bayer New Zealand Ltd, Auckland, New Zealand) and local subcutaneous analgesia (lignocaine hydrochloride 2%, Ethical Agents, Auckland, New Zealand) for infusions and blood sampling. Two catheters were implanted in 1 side for glucose and insulin infusion and 2 in the opposite side for blood sampling. In addition, the cows were fitted with the harnesses and chutes required to collect the faeces and urine for a nitrogen balance.

The jugular catheters were inserted into the cows using the method described in Chapter 3 except for the following changes. Two small sutures held the catheter in place at the entry puncture and then the catheter was attached to the neck by 3 cm strips of fabric strapping (Elastoplast) glued 10 cm apart. To protect the catheters when the cows were in the paddock and head stocked in the barn, the catheters were placed in a waterproof pouch on the outside of a canvas collar that was used instead of the elastic netting.

Catheters were kept patent between samplings using heparinised saline (chapter 3). By the end of the first HEC, it was difficult to keep some of the blood sampling catheters patent. Therefore all catheters were removed before putting the cows out to pasture for the rest period. The day before the cows returned for the second infusion period, new catheters were inserted using the method described previously.

4.3.2 Experimental Procedure

Each cow was randomly allocated to a treatment group in a 2 period cross-over design; where each animal was subjected to a HEC twice, with or without an abomasal infusion of casein. The experiment consisted of two 12 day periods, where (in each period) the first 4 days allowed acclimatisation to casein or control (buffer) infusions, with measurements being taken during days 5 to 8 to evaluate the effect of the casein infusion. All cows were then subjected to a HEC from day 9 until day 12 to allow a comparison of the effect of the insulin infusion. There was a rest period of 6 days between the 2 experimental periods to allow for any residual effects of insulin, glucose or casein to be eliminated. Cows were grazed outdoors on pasture during the rest period and brought indoors twice daily for milking.

During the baseline and HEC periods, a nitrogen balance was run. However, collections were not successful as there were problems keeping the faeces and urine separate and no results are presented in this chapter.

4.3.3 Infusions

Casein (310 g/cow/d, alacid 30 mesh acid casein, #7010-1333-N4194-1146, New Zealand Dairy Board) was infused directly into the abomasum in a 0.1 M Na phosphate buffer (pH 7.0, Scientific Supplies (NZ) Ltd). Control cows received an equal volume of the 0.1M Na phosphate buffer. The casein suspension was pumped through an infusion line fed through the rumen fistula and held in place in the abomasum by a 10 cm plastic disc (made from 6mm plastic sheeting). The infusions continued for the entire 12 d experimental period at approximately 3.6 ml/min (approx. 12.5 g casein/h) and lines were checked daily by feel that they were still in place in the abomasum.

For the HEC (on days 9-12) bovine pancreatic insulin (Sigma Chemicals, St Louis, MO.) was administered constantly (1 μg/kg BW/h), in a sterile filtered 0.5% bovine serum albumin solution (Immuno Chemical Products (NZ) Ltd). Blood glucose concentrations were monitored using an Advantage Blood Glucose Meter (Boehringer Mannheim (NZ) Ltd). This allowed rapid determination of blood glucose concentrations for adjusting the glucose infusion to maintain euglycaemia during the insulin infusion. Once circulating glucose concentrations began to decrease in response to the insulin infusion, the glucose infusion was started at half glucose entry rate for dairy cows as determined by Bickerstaffe *et al.* (1974). A sterile 45% w/w glucose solution (prepared using food grade dextrose monohydrate (Pure Chem Co. Ltd, Thailand) and autoclaving for 20 min) was infused via variable speed peristaltic pumps.

4.3.4 Sample collections, analyses and calculations

4.3.4.1 Milk

The cows were milked twice daily at 0730 and 1930 h, yields were weighed and a sample stored at -20°C for further analysis. Milk samples for somatic cell counts (SCC) were taken to monitor udder health during the rest periods, on day 2 of the baseline period (day 6 of each 12 day period), days 2 (day 10) and 4 (day 12) of the HEC. Cell count analyses were performed by Livestock Improvement Corporation (Hamilton, New Zealand).

Milk composition was estimated by near-infrared spectroscopy (NITS, in transmission mode, model 6500, NIRsystems Inc, Silver Spring, MD, USA) with PC software by Infrasoft International (version 3.1). The NITS was calibrated for cow's milk using the following analyses: Crude protein was calculated as (Total N x 6.38), with Total N being determined by combustion on a Carlo Erba NA1500 Nitrogen analyser. Non-protein nitrogen (NPN) was determined using IDF standard 20B, modified for determining ammonia by flow injection analysis. True protein was then calculated as Total N - NPN x 6.38. Fat content was determined gravimetrically following the extraction of the fat by diethyl ether (modified AOAC method 963.15, AOAC 1990). Lactose concentration was determined by HPLC, using an anion-cation exchange resin column (Aminex HPX-87P) at a temperature of 60°C with a water mobile phase and a

flow rate of 0.6 ml/min. Lactose was detected using a Waters refractive index detector (for full method, see Appendix A).

Individual casein and whey proteins were quantified by HPLC. 200 µl of skim milk and 600 µl of reconstitution buffer (200 µl each of 6 M guanidine-HCl, 0.1 M Bis-Tris, 5.5 mM trisodium citrate) were vortexed and allowed to stand at room temperature for 1 h. 500 µl of this mixture were added to 500 µl 4.5 M guanidine-HCl and 10 µl mercaptoethanol, then centrifuged (21 000 g for 5 min) and 400 µl pipetted into autosampler vials. Casein and whey protein concentrations were determined on a Shimadzu LC10/A HPLC system. An injection volume of 50 µl was used with an elution system of 2 mobile phases, buffer A (0.1 % TFA in MQ H_20) and buffer B (90% CH_3CN, 10% buffer A). A separation gradient started with 27% buffer B increasing to 33 % at 2 min, increasing to 48.4 % at 36.9 min, then to 50.2 % at 40.9 min, and ending with a dump step of an increase to 90 % at 43 min, dropping to 27 % at 46 min where it remained until the run ended at 68 min. The flow rate was set at 1.0 ml/min. Eluents were kept under a blanket of $He_{(g)}$ to remove N_2 and 0_2 from solution. Separation was on a Phenomenex Jupiter column (250 x 4.6 mm) with an oven temperature of 33 °C. Eluted proteins were detected with a UV detector set at 254 nm.

The recovery of the three individual caseins with this method was over-estimated because of the impurity of standards used. Therefore, the weights were corrected for the specific gravity of the buffers used and the N content, DM and standard purity reported on the label by the manufacturer to try to account for this.

Milk minerals were measured in acid digested skim milk samples. Approximately 3 ml of skim milk were steeped overnight in 10 ml concentrated HNO_3. The following morning the mixture was gently heated (<80°C) until all samples ceased to emit NO_2 fumes and the samples were colourless. The samples were then heated at 120°C until nearly dry. The residue of each sample was then dissolved in 2M HCl and this solution analysed for milk minerals by inductive coupled plasma emission spectrometry (ICP) on an ARL 34000 ICPES (Lee 1984).

Individual milk fatty acids of chain lengths C_4 to $C_{18:3}$ were measured (DRI, Palmerston North, New Zealand). Methyl esters were prepared by direct trans-esterification of an anhydrous fat sample with sodium methoxide/methanol. Individual fatty acids were determined by gas chromatography of the methyl esters (Christopherson & Glass 1969, Bannon *et al.* 1986, Richardson 1989). Gas chromatography was conducted on a Shimadzu 17A system, with an Alltech EC-1000 column (15m x 0.53 mm, 1.2 μm film thickness) with a 1m deactivated FSOT as a pre-column. A sample volume of 1 μl was injected into a H_2 (10 ml/min) mobile phase. Fatty acids were detected using a flame ionisation detector at 250°C.

4.3.4.2 Blood

During days 5-9 of each 12 day experimental period, blood samples were taken at 1000, 1200 and 1400 to establish basal concentrations of glucose, insulin, non-esterified fatty acids (NEFA), β-hydroxybutyrate, triacylglyerols, insulin-like growth factor-I (IGF-I) and amino acids (AA). An average glucose concentration was determined, which was the target euglycaemia (± 10%) for each individual animal during the subsequent HEC. To assess the effect of insulin on circulating concentrations of insulin, glucose, NEFA, triacylglyerols, IGF-I, AA and β-hydroxybutyrate, blood samples were taken over the 4 days of the HEC. During the first 24 h, blood samples were taken at 1000, 1200, 1400, 2000, 0200, and 0800 h. Thereafter, on days 2, 3 and 4 of the HEC, samples were taken at 1000, 1200 and 1400 h.

All blood samples were collected with disodium ethylenediaminetetraacetate (Na_2.EDTA) as the anticoagulant. A subsample (1 ml) of whole blood was deproteinised by addition of 0.5 ml of 30% trichloroacetic acid (TCA), centrifuged at 3270 g at 4°C for 15 min and filtered through a 0.45μm cellulose syringe filter. This filtrate was used to measure circulating AA concentrations by two methods: Amino acid concentrations (exclusive of cysteine) were determined by reverse phase HPLC separation of phenylisothiocyanate derivatives on a Waters PicoTag© column used in conjunction with a Shimadzu LC10/A HPLC system using a modified method of Bidlingmeyer *et al.* (1984) (for full method see Appendix B). Cysteine concentrations were determined by a modified automated method using acid ninhydrin (Gaitonde 1967). Concentrations of tryptophan are not reported because of the uncertainty in the

proportion of tryptophan associated with plasma protein that is released by the extraction method. The remaining blood was centrifuged (at 3270 g at 4°C for 15 min) and the resulting plasma was harvested and stored at -20°C until analysed. Amino acid concentrations are grouped as essential (EAA) or non-essential (NEAA) as defined for lactating dairy cows by Clark *et al.* (1977).

Plasma insulin concentrations were measured using a double antibody radioimmunoassay (RIA) (Flux *et al.* 1984). Intra- and inter-assay coefficients of variation were 8.7% and 12.9% respectively. The mean sensitivity was 22.7 pg insulin/ml. Plasma IGF-1 concentrations were determined by RIA using an acid/ethanol procedure as reported by Prosser *et al.* (1995).

Plasma cortisol concentrations were also measured using a RIA (as described by T. Manley per. com.). Duplicate samples or standards (made in charcoal stripped bovine plasma) were incubated for 24 h at 4°C with iodinated tracer (Diagnostic Products Corporation Los Angeles, CA) and antiserum (raised in a New Zealand white rabbit against 4-pregnen-11β, 17, 21 - triol - 3, 20 - dione 3 - CMO :BSA) at an initial tube dilution of 1:5000. On day 2 sheep anti-rabbit second antibody was added and the tubes were incubated overnight at 4°C. On day 3, polyethylene glycol 6000 (8%W/V) was added before centrifuging at 1800g for 35 min. Dilutions of a plasma sample with a high cortisol concentration in plasma charcoal stripped plasma were parallel to the standard curve and the sensitivity of the assay was 2.0 ng/ml. For a mean cortisol concentration of 8.7 ng/ml, the intra-assay CV was 14.1%.

Plasma NEFA and β-hydroxybutyrate concentrations were measured by enzymatic colorimetric methods that have been adapted to a Cobas Fara II autoanalyser (Hoffman-La Roche Ltd, Basal, Switzerland). The NEFA method was based on that of McCutcheon & Bauman (1986), utilising a Wako C test kit (Wako Pure Chemical Industries Ltd, Osaka, Japan) and the intra- and inter- assay coefficients of variation were 4.6% and 2.3% respectively. The β-hydroxybutyrate method was based on that of Williamson & Mellanby (1974), with the intra- and inter- assay coefficients of variation being 4.7% and 2.1% respectively. The triacylglyerol method was based on that of

McGowan *et al.* (1983) and Fossati & Prencipe (1988). The intra- and inter- assay coefficients of variation were 2.4 and 2.7% respectively.

4.3.4.3 Feed intake

The cows were fed four times daily and the feed offered was recorded. Refusals from each feed were collected from the bins and floor, bulked separately, weighed and the bin refusals subsampled for analysis. Dry matter was calculated on both feed offered and refused and daily dry matter intake (DMI) was measured as offered less refused. Daily samples were analysed for crude protein (CP) and energy (ME) by NIRS calibrated for pasture (Corson *et al.* 1999).

Feed intake was also predicted using an iterative algorithim developed from equations 1.21 and 1.24 in "Feeding Standards for Australian Livestock CSIRO" (1990):

$$ME_m\,(MJ.d^{-1}) = \frac{1.4 \bullet 0.28W^{0.75} \bullet \exp^{-0.03A}}{K_m + 0.1ME_P + EGRAZE \bullet K_m^{-1}} \qquad 1.21$$

$$EGRAZE\,(MJ\ net\ energy \bullet d^{-1}) = W\left(0.006 \bullet DMI \bullet (0.9 - D) + \frac{0.05}{GF} + 3\right) \qquad 1.24$$

where W = live weight, A = age in years (used 4), K_m = net efficiency of use of ME for maintenance, ME_p is the amount of dietary energy directly used for production, DMI = dry matter intake from pasture (used actual intakes measured in the experiment), DM = digestibility (used period 1 = 0.604, period 2 = 0.719), and GF = availability of green forage to ground level (tonnes DM/ha). To use this algorithm, an initial estimate of intake was given (used the actual intake from the experiment). The total ME for maintenance plus specified production was then calculated from experimental data from each animal (W, milk yield and composition, pasture characteristics etc). Liveweight change was not used due to confounding effect of reduced intake and change in gut fill in this experiment. This value was divided by the known ME content of the forage to provide an updated estimate of intake and the procedure then iterated.

4.3.5 Statistical analysis

The two experiments were conducted with a 2 period cross-over design, with 8 cows being housed indoors for each experimental period. Six cows were involved in the main HEC experiment and 2 in the chromium supplementation trial that was run

concurrently. During the main HEC experiment 2 cows did not acclimatise to being housed indoors. One cow was replaced when the cows were housed for the second experimental period by 1 of the chromium treated cows from the first period. The rationale behind the substitution was that, as the substitute cow was in the trial being run concurrently during the first period (but infused with chromium, not control buffer), she had undergone similar treatment as the cow that was replaced, as all cows were subjected to the HEC. A one-way ANOVA for the first experimental period showed no significant differences between the cows on control and chromium treatments. This allowed the experiment to continue with 6 cows. However, during the second period of infusion, a second cow had problems sitting and standing in the stalls, and her data were subsequently dropped from the cross-over analyses and used only in the chromium trial analyses. Therefore data were analysed with a split-plot analysis with repeated measures. The casein treatment was considered as the main plot, with the HEC as the split-plot and time was used as the repeated factor. Analyses were performed using the procedure GLM from the statistical package SAS (1988). The model used for the analyses was:

$$Y_{ijkl} = \mu + \delta_k + \pi_{(l)k} + \alpha_i + \beta_j + \varepsilon_{ijkl}$$

where μ is the mean, δ_k is the fixed effect due to the group k, α_i is the fixed effect due to the treatment i, β_j is the fixed effect due to period j, and $\pi_{(l)k}$ is the l^{th} cow in group k.

Results are expressed as least squares means ± standard errors. All treatment and period effects and their interactions were tested. Differences between treatments were assessed by a multiple comparison test (PDIFF option of Proc GLM, SAS Institute 1996) of the calculated least squares means. Significance levels of $P<0.05$ were taken to indicate significant effects, whereas levels of $0.05 < P < 0.1$ were considered to indicate trends. Normality of the data was tested by plotting the standardised residuals against the standardised predicted values of the response variables. No plots showed a pattern that would indicate that the normality assumption of the ANOVA model should be questioned and that the data should be transformed.

4.4 Results

Abomasal infusion of casein did not change concentrations of plasma insulin and blood glucose or the amount of glucose required to maintain euglycaemia (Table 4-1).

Circulating plasma insulin concentrations significantly increased in both the casein infused and control cows during the HEC but to a significantly greater extent in control cows.

Overall, blood glucose concentrations were maintained within 10% of the range determined for euglycaemia during casein infusion period in the casein infused cows. However, the concentration of blood glucose in the control cows was slightly but significantly above this range (Table 4-1).

Table 4-1 Plasma insulin and blood glucose concentrations (LSMeans ± SEM) on day 4 of the infusion period and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC	
	Casein	Control	Casein	Control
Insulin (pg/ml)	265 (±487)^{a}	388 (±494)^{a}	3860 (±780)^{b}	6653 (±712)^{c}
Blood glucose (mM)[1]	3.3 (±0.2)^{a}	3.4 (±0.2)^{a}	3.5 (±0.2)^{a}	4.1 (±0.2)^{b}

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused (n=5), control = buffer infused (n=5).
LSMeans with different superscripts are significantly different at $P<0.05$.
[1] Values for infusion day 4 in both casein and control cows are an average calculated from samples taken over the 4 days of the infusion period.

The amount of glucose required to maintain euglycaemia as measured by the glucose infusion rate, increased significantly over the 4 days of HEC in both the casein infused and control cows ($P<0.001$). In the casein infused cows the glucose infusion rate increased from 0.68 ± 0.11 g/min on day 1 to 1.34 ± 0.11 g/min on day 4, whereas it increased from 0.67 ± 0.11 g/min to 1.18 ± 0.11 g/min in the control cows.

Abomasal infusion of casein had no effect on feed intake and consequently no effect on dietary crude protein (CP) or ME intake. Feed intake fell gradually over the 4 days of the HEC so that by the final day it was significantly lower than on day 4 of the infusion period (Table 4-2). There was also a significant decrease in dietary CP intake but not ME intake in both the casein infused and control cows. There was a significant effect of

period of insulin infusion on feed intake (P<0.01) because of a larger reduction of intake during the first period of insulin infusion (average intake in period one was 9.07 ± 0.31 kg DM/d versus period two, 13.70 ± 0.31 kg DM/d). This pattern was also shown in dietary CP and ME intakes. Crude protein intake was significantly higher (P<0.05) in the second period of infusion (1815 ± 58 g CP/d vs 2022 ± 58 g CP/d) and ME intake was also significantly higher (P<0.01) in the second period (105.3 ± 35 MJ ME/d vs 147.2 ± 35 MJ ME/d). Pasture quality, on a CP basis, changed between the periods of infusion. For the first period, average pasture CP content was 18.7 % compared to 14.7 % in the second period. ME content was 11.0 % per kg DM for both periods.

Table 4-2 Comparison of feed intakes and energy balance (LSMeans ± SEM) on day 4 of the casein infusion and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC		
	Casein	Control	Casein	Control	± SEM
DMI (kg/d)	10.93^{ab}	12.38^{a}	9.83^{b}	9.31^{b}	0.85
CP (g/d)	2092^{a}	2134^{a}	1360^{b}	1299^{b}	160
Total CP (g/d)[1]	2402^{a}	2134^{a}	1670^{b}	1299^{b}	160
ME (MJ ME/d)	127.9	141.4	107.4	102.2	9.82
Total ME (MJ ME/d)[2]	135.6	141.1	142.3	129.0	10.4
Efficiency of CP utilisation (g/g)[3]	0.23^{a}	0.21^{a}	0.49^{c}	0.38^{bc}	0.06

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused (n=5), control = buffer infused (n=5).
LSMeans with different superscripts are significantly different at P<0.05.
[1, 2] Total energy (MJ ME) and crude protein intakes were calculated by combining dietary intake with additional ME or CP available from the glucose and casein infusions
[3] Calculated as g milk protein produced per g of CP intake

Total crude protein and energy supplied to the cows by the casein and glucose infusions during the HEC were calculated by adding the amounts supplied by the infusions to dietary CP and ME intakes (Table 4-2). There was no difference in total CP or ME intakes between the casein infused and control cows on day 4 of the infusion period. However, on the fourth day of the HEC, the dietary CP intakes of the casein infused and control cows were significantly lower than during the casein infusion.

The efficiency of CP utilisation is a measure of the gross efficiency of dietary CP utilisation for milk protein production. Casein infused and control cows had a similar utilisation (as shown in Table 4-2) on day 4 of the casein infusion. However, efficiency of utilisation increased significantly in casein infused and control cows on day 4 of the HEC.

Feed intake during the experimental periods was compared to intakes predicted by a feed intake model using an algorithm as described in the materials and methods section. The following data presented in Table 4-3 are raw data and have not been subjected to statistical analysis and therefore have no error term. The comparison shows that for the level of production during the first period of infusion the cows did not eat enough for the level of milk production. However, this changed during the second period of infusion, when cows ate more than was required for their level of milk production.

Table 4-3 Comparison of actual and predicted feed intakes and milk yield on day 4 of the infusion period and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC) during both periods of infusion.

	Casein infusion		HEC	
	Casein	Control	Casein	Control
Period 1	n=3	n=2	n=3	n=2
Actual milk yield (kg/d)	16.8	14.9	15.7	9.7
Actual feed intake (kg DM/d)	10.2	8.7	6.4	3.8
Predicted feed intake (kg DM/d)	14.7	12.1	12.0	9.2
Period 2	n=2	n=3	n=2	n=3
Actual milk yield (kg/d)	13.2	13.2	12.2	9.8
Actual feed intake (kg DM/day)	11.2	15.8	14.2	14.0
Predicted feed intake (kg DM/day)	10.9	11.4	8.8	10.8

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused group, control = buffer infused group.

Abomasal infusion of casein infusion had no effect on milk yield, as there was no difference in production between the casein infused and control cows on day 4 of the casein infusion (Table 4-4). On day 4 of the HEC milk yield of the control cows was significantly lower and tended (P=0.08) to be lower in the casein infused cows. Milk yield of the control cows was not significantly different from that of the casein infused cows over the first 3 days of the HEC but dropped significantly on the fourth day (data not presented). This pattern was demonstrated in both periods of insulin infusion. There was a significant effect of period of infusion (P<0.001) on milk yield, as yield was lower during the second period of infusion.

Table 4-4 Comparison of milk yield, yield and concentration of milk components (LSMeans ± SEM) on day 4 of the infusion period and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Casein infusion		HEC		
	Casein	Control	Casein	Control	± SEM
Milk yield (kg/d)	14.3^{a}	14.3^{a}	13.2^{a}	9.9^{b}	0.5
Component yield					
Total solids (kg/d)[1]	2.14^{a}	1.80^{b}	1.69^{b}	1.25^{c}	0.07
Crude protein (g/d)	482^{a}	421^{b}	459^{ab}	311^{c}	16
Fat (g/d)	810^{a}	649^{b}	522^{c}	419^{c}	40
Lactose (g/d)	708^{a}	665^{ab}	607^{b}	440^{c}	25
Concentration (%)					
Total solids[1]	14.1^{a}	12.9^{b}	12.5^{b}	11.6^{c}	0.3
Crude protein	3.2^{ab}	3.1^{a}	3.3^{b}	3.3^{b}	0.1
Fat	5.3^{a}	4.7^{ab}	3.8^{c}	4.5^{b}	0.2
Lactose	4.7^{a}	4.8^{a}	4.3^{b}	4.5^{b}	0.1
Individual Milk Proteins (mg/ml)					
α_{s1}-casein	14.8^{a}	15.4^{a}	13.7^{a}	11.9^{b}	0.7
β-casein	11.2	11.7	10.5	10.5	0.7
κ-casein	3.7	3.6	4.3	3.8	0.4
α-lactalbumin	0.98^{ab}	1.1^{a}	0.99^{ab}	0.79^{b}	0.08
β-lactoglobulin B	2.5^{a}	2.3^{a}	2.5^{a}	1.7^{b}	0.2

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused (n=5), control = buffer infused (n=5).
LSMeans with different superscripts are significantly different at P<0.05.
[1]Total solids = total solids in milk as determined by freeze drying.

Casein infused cows had a significantly greater milk crude protein and fat yield than control cows (Table 4-4). Crude protein yield was significantly reduced in the control cows by the HEC. However, crude protein concentration was significantly higher in the control cows on day 4 of the HEC. While crude protein yield was influenced by period of infusion ($P<0.001$), concentration was not.

The HEC significantly reduced milk fat yield in both the casein infused and control cows whereas milk fat concentration was significantly lower in the casein infused cows on day 4 of the HEC. Milk fat yield ($P<0.001$) and concentration ($P<0.001$) were both significantly influenced by period of infusion.

Abomasal infusion of casein had no effect on milk lactose yield and total solids yields. On day 4 of the HEC, lactose and total solids yields had significantly decreased and a similar pattern was shown in lactose and total solids concentration. While lactose yield ($P<0.001$), total solids yield ($P<0.001$), and concentration ($P<0.001$), were significantly affected by period of infusion, lactose concentration was not.

Abomasal infusion of casein had no effect on concentrations of α_{s1}-, β- or κ-casein (see Table 4-4). The HEC significantly reduced concentrations of α_{s1}-casein in the control cows, whereas there was no significant change in β-casein concentrations. For the κ-casein there was a numerical increase in both the casein infused and control cows on day 4 of the HEC. There was no effect of period of infusion on α_{s1}-casein concentrations, whereas there tended ($P=0.08$) to be a period of infusion on β-casein concentrations. There was a significant period of insulin infusion effect on κ-casein concentration ($P<0.001$) as concentrations were greater in the second period of infusion. There was no significant difference in concentrations of α-lactalbumin and β-lactoglobulin B whey proteins in casein infused and control cows on day 4 of the casein infusion. While concentrations did not significantly change in the casein infused cows on day 4 of the HEC, they were significantly lower in the control cows. There was no period of insulin infusion effect on either whey protein.

Milk yield and composition, and feed intake were also monitored for the 2 days post HEC, before the cows were returned to pasture. On the day immediately after cessation of the HEC, milk yield and feed intake returned to a similar level as the casein infusion and first 2 days of the insulin clamp (data not presented). During this time, milk crude protein concentration increased significantly to 3.9 ± 0.1 % in both casein infused and control cows on the day immediately after the cessation of the HEC. It then decreased to 3.5 ± 0.1 % in the casein infused and 3.4 ± 0.1 % in the control cows on the following day. Of the individual milk proteins measured, the main shift was in the κ-caseins, which significantly increased in the HEC ewes from 4.3 ± 0.4 on the final day to 5.4 ± 0.4 mg/ml, whereas in the control ewes it increased from 3.8 ± 0.4 to 5.0 ± 0.4 mg/ml.

Circulating concentrations of AA were measured on day 4 of the infusion period, during the first 24 h and on day 4 of the HEC. Abomasal infusion of casein had no significant effect on circulating concentrations of most EAA except for phenylalanine and histidine (Table 4-5). Concentrations of phenylalanine were significantly higher in the control cows whereas concentrations of histidine were significantly lower. Of the NEAA measured, there were no significant differences in circulating concentrations of AA on day 4 of the infusion period.

The HEC caused a significant decrease in circulating concentrations of all EAA except methionine (Table 4-5). In the NEAA measured, all concentrations decreased except for glycine, which increased in the casein supplemented cows. There was no period of infusion effect on circulating concentrations of AA except for cysteine ($P<0.05$) and histidine ($P<0.05$). There was no effect of sequence of infusion on circulating AA concentrations.

Table 4-5 Circulating concentrations (LSMeans ± SEM) of amino acids in plasma on day 4 of the infusion period and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC	
	Casein	Control	Casein	Control
Essential amino acids (μM)				
Arginine	84.9 (± 5.1)^{a}	96.4 (± 5.1)^{a}	27.0 (± 5.6)^{b}	25.1 (± 5.6)^{c}
Cysteine	112.5 (± 4.0)^{a}	115.6 (± 3.9)^{a}	86.5 (± 4.2)^{b}	73.1 (± 4.2)^{b}
Histidine	43.9 (± 1.7)^{a}	34.9 (± 1.7)^{b}	30.3 (± 1.8)^{b}	23.9 (± 1.8)^{c}
Isoleucine	97.7 (± 9.8)^{a}	102.2 (± 9.8)^{a}	66.2 (± 10.7)^{b}	74.5 (± 10.6)^{ab}
Leucine	115.1 (± 7.5)^{a}	108.1 (± 7.5)^{a}	36.0 (± 8.2)^{b}	34.0 (±8.1)^{b}
Lysine	96.2 (± 6.4)^{a}	92.5 (± 6.4)^{a}	27.7 (± 8.7)^{b}	33.0 (± 9.1)^{b}
Methionine	26.4 (± 4.9)	28.8 (± 5.1)	25.0 (± 4.7)	15.6 (± 5.1)
Phenylalanine	42.3 (± 3.7)^{a}	56.5 (± 3.7)^{b}	26.2 (± 4.0)^{c}	23.4 (± 4.0)^{c}
Threonine	83.9 (± 5.1)^{a}	91.9 (± 5.1)^{a}	45.9 (± 5.6)^{b}	37.2 (± 5.8)^{b}
Tyrosine	43.1 (± 3.2)^{a}	43.3 (± 3.2)^{a}	22.9 (± 3.5)^{b}	22.0 (± 3.4)^{b}
Valine	207.4 (± 1.9)^{a}	188.2 (± 0.9)^{a}	49.5 (± 4.7)^{b}	46.7 (± 3.6)^{b}
Non essential amino acids (μM)				
Alanine	110.3 (± 7.4)^{a}	127.9 (± 7.4)^{a}	73.6 (± 8.1)^{b}	52.0 (± 8.4)^{b}
Glycine	185.4 (± 56.6)^{a}	399.3 (± 56.6)^{a}	265.3 (± 61.2)^{b}	331.3 (±61.1)^{ab}
Proline	68.9 (± 3.5)^{a}	60.3 (± 3.2)^{a}	34.3 (± 3.4)^{b}	23.4 (± 3.4)^{c}
Serine	75.4 (± 4.3)^{ab}	82.9 (± 4.3)^{a}	79.3 (± 4.7)^{ab}	69.8 (± 4.6)^{b}

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused (n=5), control = buffer infused (n=5).
LSMeans with different superscripts are significantly different at $P<0.05$.

Changes in circulating concentrations of amino acids were compared between day 4 of the infusion period, after the first 24 h and on day 4 of the HEC. While circulating concentrations of most EAA (except methionine) significantly decreased over the 4 days of the HEC, there appeared to be a different effect on individual AA as the severity and rate of decline differed. There were significant decreases in EAA concentrations over the first 24 h and a further significant decrease on day 4 of the HEC in both the casein and control cows for threonine, lysine, arginine, valine and leucine. Isoleucine significantly decreased over the first 24 h in both groups of cows, but concentrations did not decrease further over the remaining 3 days of the HEC. The concentrations of BCAA had decreased on average by 58% on day 4 of the HEC, and threonine, arginine and lysine by similar amounts (on average 67%). Of the other EAA, phenylalanine concentrations in both the casein infused and control cows showed a non-significant decrease over the first 24 h, but were significantly lower on day 4 of the HEC.

Tyrosine, histidine and cysteine showed the same pattern of change over the 4 days of the HEC. In the casein infused cows, concentrations significantly decreased over the first 24 h, whereas the decrease in the control cows was non-significant. However, concentrations in both the casein infused and control cows were significantly lower on day 4 of the HEC.

Plasma concentrations of those NEAA that were measured are presented in Table 4.5. There was no significant change in serine concentrations in the casein infused cows, compared to the control cows where concentrations had significantly decreased by 15% by day 4 of the HEC. There was no significant change in glycine concentrations in either casein infused or control cows. Alanine concentrations decreased non-significantly over the first 24 h of the HEC, but were significantly reduced on day 4 in casein infused and control cows. Proline concentrations significantly decreased over the first 24 h in casein supplemented and control cows. Concentrations were further reduced on day 4 of the HEC, so that they were 55% lower than baseline concentrations.

Abomasal infusion of casein had no effect on circulating plasma concentrations of NEFA, β-hydroxybutyrate and triacylglyercol (Table 4-6). Concentrations of NEFA and β-hydroxybutyrate were significantly reduced over the first 24 h of the HEC in both the casein supplemented and control cows and were measured at a similar concentration for the following 3 days, except for NEFA concentration in the control cows, which significantly decreased further. The HEC did not alter triacylglycerol concentrations although there tended to be ($P=0.08$) a difference between concentrations during the 2 periods of infusion. There was a significant period of insulin infusion effect on NEFA ($P<0.001$) and β-hydroxybutyrate ($P<0.001$) concentrations as concentrations were lower in the second period of infusion.

Table 4-6 Comparison of plasma metabolite concentrations (LSMeans ± SEM) on day 4 of the infusion period and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC	
	Casein	Control	Casein	Control
NEFA (μequiv/ml)	148 (± 14)[a]	160 (± 13)[a]	50 (± 13)[b]	53 (± 13)[c]
β-hydroxybutyrate (mM)	0.65 (± 0.03)[a]	0.60 (± 0.03)[a]	0.42 (± 0.03)[b]	0.37 (± 0.03)[b]
Triacylglycerols (mM)	0.09 (± 0.01)	0.11 (± 0.01)	0.11 (± 0.01)	0.10 (± 0.01)

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused (n=5), control = buffer infused (n=5).
LSMeans with different superscripts are significantly different at $P<0.05$.

The HEC caused significant changes in concentrations and yields of milk fatty acids (Tables 4-7 and 4-8). A small number of samples were analysed for milk fatty acid composition and as a result of this, only an effect of HEC was tested by t-test. Therefore, significance values should be treated with caution. The concentration of most chain lengths less than $C_{17:0}$ increased (Table 4-7), except for $C_{4:0}$ which decreased and $C_{10:1}$, $C_{14:0}$ and $C_{15:0}$ where there was no change. For chain lengths $C_{17:0}$ and greater, concentrations were reduced except for $C_{18:2\ conj}$ where there was an increase.

Table 4-7 Comparison of milk fatty acids concentrations (LSMeans ± SEM) on day 4 of the casein infusion and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Control[1]	HEC[1]	± SEM	P value
Concentration (%)				
$C_{4:0}$	3.58	3.23	0.09	0.05
$C_{6:0}$	2.18	2.45	0.04	0.01
$C_{8:0}$	1.30	1.60	0.04	0.01
$C_{10:0}$	2.73	4.18	0.17	0.001
$C_{10:1}$	0.35	0.60	0.12	ns
$C_{12:0}$	3.03	5.58	0.31	0.01
$C_{14:0}$	10.85	11.63	0.41	ns
$C_{14:1}$	0.63	1.40	0.06	0.001
$C_{15:0}$	1.2	1.2	0.03	ns
$C_{16:0}$	28.10	37.08	1.46	0.01
$C_{16:1}$	1.73	2.33	0.23	ns
$C_{17:0}$	0.50	0.43	0.02	0.05
$C_{18:0}$	15.0	7.30	0.99	0.01
$C_{18:1}$	23.80	16.15	1.01	0.01
$C_{18:2}$	1.18	0.90	0.0	0.05
$C_{18:2conj}$	0.70	0.90	0.06	0.05
$C_{18:3}$	0.85	0.53	0.05	0.01

[1]Treatments: control = average of casein infused and buffer infused cows on day 4 of the infusion period (n=4), HEC = average of casein and control cows on day 4 of the hyperinsulinaemic euglycaemic clamp (n=4).

Milk fatty acid yields are presented in Table 4-8. For chain lengths less than $C_{17:0}$, milk fatty acids yields decreased for $C_{4:0}$, $C_{6:0}$, $C_{15:0}$ and $C_{16:0}$. There was no change in yield for most of the medium chain fatty acids except for $C_{14:1}$, which increased. For yields of chain lengths $C_{17:0}$ and greater, all were reduced except for $C_{18:2conj}$ which was unchanged.

Table 4-8 Comparison of milk fatty acid yields (LSMeans ± SEM) on day 4 of the infusion period and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Control[1]	HEC[1]	± SEM	P value
Yield (g/d)				
$C_{4:0}$	22.0	14.5	1.6	0.05
$C_{6:0}$	13.4	11.0	1.1	ns
$C_{8:0}$	8.0	7.1	0.7	ns
$C_{10:0}$	18.6	16.7	1.8	ns
$C_{10:1}$	2.1	2.8	0.8	ns
$C_{12:0}$	18.6	24.8	2.5	ns
$C_{14:0}$	66.6	51.8	5.4	ns
$C_{14:1}$	3.8	6.3	0.7	0.05
$C_{15:0}$	7.4	5.4	0.6	(0.06)
$C_{16:0}$	172.6	166.2	19.7	ns
$C_{16:1}$	10.7	10.6	1.9	ns
$C_{17:0}$	3.1	1.9	0.3	0.05
$C_{18:0}$	92.4	32.1	7.0	0.001
$C_{18:1}$	146.4	72.2	10.0	0.01
$C_{18:2}$	7.24	3.92	0.5	0.01
$C_{18:2conj}$	4.3	4.0	0.5	ns
$C_{18:3}$	5.3	2.4	0.5	0.01

[1]Treatments: control = average of casein infused and buffer infused cows on day 4 of the infusion period (n=4), HEC = average of casein and control cows on day 4 of the hyperinsulinaemic euglycaemic clamp (n=4).

Circulating concentrations of plasma IGF-1 and cortisol on day 4 of the infusion period and day 4 of the HEC are presented in Table 4-9. Plasma IGF-I concentrations were increased significantly by HEC in both the casein supplemented and control cows, whereas cortisol concentrations did not change significantly in the casein supplemented cows but were significantly higher on day 4 in the control cows. There was no effect of sequence or period of infusion on either IGF-1 or cortisol concentrations.

Table 4-9 Comparison of circulating concentrations (LSMeans ± SEM) of IGF-1 and cortisol in plasma on day 4 of the infusion period and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Infusion		HEC	
	Casein	Control	Casein	Control
IGF-1 (ng/ml)	52 (± 5)[a]	49 (± 4)[a]	78 (± 4)[b]	87 (± 5)[b]
Cortisol (ng/ml)	6.8 (± 2.0)[a]	6.6 (± 1.9)[a]	6.2 (± 1.9)[a]	12.0 (± 1.9)[b]

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused (n=5), control = buffer infused (n=5).
LSMeans with different superscripts are significantly different at $P<0.05$.

Somatic cell counts were not significantly different between the casein infused and control cows on any day tested. Although SCC increased in all cows between day 2 and 4 of the HEC during both periods of infusion, the counts were greater than 200,000 cells/ml (the industry standard for mastitis detection) on day 4 of the second HEC. Counts increased from 22 800 ± 24 207 cells/ml (average ± SD) on day 2 to 1 776 400 ± 947 004 on day 4 of the second HEC. As a result of this, there tended ($P=0.087$) to be a period effect. However, this did not develop into mastitis as SCC were below the 200 000 level 4 days after cessation of the HEC.

Neither abomasal infusion of casein or the HEC had any effect on concentration of potassium or phosphorus in milk (Table 4-10). For milk concentrations of calcium, magnesium, sodium, and sulphur, infusion of casein had no effect on concentrations. However, on day 4 of the HEC, concentrations had increased, although this was not significant for calcium in the casein infused cows. Concentrations of sodium and sulphur in the control cows tended ($P=0.08$) to be higher. Concentrations were not influenced by sequence or period of infusion, except for sodium and sulphur, where there was a period of infusion effect ($P<0.05$ for both) because of higher concentrations in the second period of infusion.

Table 4-10 Concentrations (LSMeans ± SEM) of minerals in milk on day 4 of the casein infusion and day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Casein infusion		HEC		
	Casein	Control	Casein	Control	± SEM
Concentration (mg/ml)					
Calcium	1303[a]	1271[a]	1383[a]	1445[b]	40
Potassium	1478	1420	1512	1403	65
Magnesium	91[a]	92[a]	101[b]	112[b]	3
Sodium	333[ab]	307[a]	429[b]	399[ab]	33
Phosphorus	947	910	963	921	30
Sulphur	320[a]	328[ab]	366[b]	368[b]	14

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, casein = casein infused group, control = water infused group.
LSMeans with different superscripts are significantly different at $P<0.05$.

4.5 Discussion

The main aim of this experiment was to test if under HEC conditions, milk protein production could be increased in lactating, pasture-fed cows, and secondly, to compare the results with those obtained with lactating ewes under the same experimental conditions (Chapter 3).

As shown in Table 4-1 the insulin infusion during the HEC caused hyperinsulinaemia. However, the changes in circulating insulin concentrations caused by the HEC are very large and it is not clear why the insulin infusion rate, which is the same as that used in Chapter 3 and the US cow studies (McGuire *et al.* 1995a, Griinari *et al.* 1997a, Mackle *et al.* 1999) on a body weight basis, should cause on average a 15 fold increase. Either there was a large difference in insulin metabolism between the cows in this experiment compared with animals used in the other experiments (where there was a 3-4 fold increase) or that insulin concentrations were over-estimated by the assay. The rate of glucose disposal is similar between experiments if the amount of glucose used to maintain euglyaemia as measured by the glucose infusion rate is considered. Bequette *et al.* (2001) estimated that the amount of glucose infused per kg body weight to maintain euglycaemia across the HEC studies is relatively consistent, ranging from 4.8 – 5.8 g/kg BW in goats (Debras *et al.* 1989, Bequette *et al.* 2001), and 5.2 g/ kg BW in cows (Griinari *et al.* 1997a, Mackle *et al.* 1999). Assuming an average body weight of 350 kg for these Jersey cows, it is estimated that the glucose infused to maintain

euglycaemia was on average 5.5 g/kg BW for the casein infused cows and 4.9 g/kg BW for the control cows. The similarity in these values would appear to indicate that despite the high concentrations measured, the insulin dose rate had stimulated similar glucose disposal as other HEC studies and in reality, the actual increase in circulating concentrations may have been more like the 4 fold increases seen during other studies.

In contrast to other studies utilising the HEC (McGuire *et al.* 1995a, Griinari *et al.* 1997a, Mackle *et al.* 1999, 2000, Bequette *et al.* 2001, Chapter 3), neither the casein infusion, the HEC alone or HEC plus casein infusion increased or in the case of the control cows, maintained milk or milk protein yield in these pasture-fed cows (Table 4-4). However, as reported by Annen *et al.* (1998) not all studies using a long term (4 day) HEC have shown an increase in milk protein production. Circulating concentrations of AA were reduced and this was consistent with other HEC studies. Furthermore, the reductions were of a similar magnitude to those reported previously, so these lowered AA concentrations were not necessarily the cause of the reduced yield. Bequette *et al.* (2001) summarised that the HEC stimulated milk production when casein or AA infusion alone failed to stimulate a milk production response. The cows in this experiment do not fit this pattern, as the cows were able to maintain production for 3 of the 4 days of the HEC (data not shown). Yields of milk and milk components measured on day 4 of the HEC were lower, significantly so in the control cows (Table 4-4). There was no response to the casein infusion therefore it does not appear that the cows were protein deficient. However, casein may have been used as an energy source to support milk production when DMI was decreasing during the HEC. As shown in Table 4-3, the data analysed in this experiment were severely influenced by the period of insulin infusion. The feed intake model was used as an alternative to the energy balance calculated in Chapter 3 because the effect of the HEC on intake was different between the two periods of HEC. By the end of the first period of insulin infusion, DMI was severely reduced and was calculated by the intake model to be substantially less than was required for that level of milk production. However, during the second period of insulin infusion, the cows ate far more than was required for milk production. This is consistent with what would be expected later in lactation when body reserves would be being replenished. This stage of lactation effect is also consistent with what was observed in Chapter 3 with the ewes.

Despite decreasing milk and milk protein yields, there was a small shift in milk crude protein concentration where concentration was maintained, and slightly but significantly increased in the control cows. In addition, milk protein concentration significantly increased in both groups of cows after the HEC was stopped and k-casein concentrations increased. It is difficult to identify if this is a direct effect of the HEC and there is no other published data to compare it to. It does not appear to be an effect on increasing feed intake due to the HEC being discontinued, as the slight increase in intake was not significantly different from intake during the HEC. It may have been that during the HEC insulin stimulated increased AA uptake via transporters but this effect was obscured by the effect on intake. Bequette *et al.* (2001) reported that under HEC conditions in lactating goats, transport activity for BCAA and lysine was increased. Therefore, if insulin was having an effect on AA uptake by the mammary gland via AA transporters, it is possible that this effect would not stop as soon as insulin infusion ceased. Unfortunately, plasma insulin concentrations were not measured after the HEC was stopped, so it is not known when they returned to pre-infusion concentrations.

It must be acknowledged that the values for crude protein concentration in Jersey cow milk are low (Table 4-4) and would be expected to be around 3.5 – 4.5% (i.e. Verkerk *et al.* 1999; Thomson *et al.* 2001) in New Zealand Jersey cows. Values for these cows pre-trial when they were grazing pasture ranged from 3.53 – 3.93%. The lower milk protein concentrations may have been caused by the experimental conditions of housing pasture-grazing animals indoors. The cows had a 2 week training period before the experiment commenced but this may not have been sufficient. Housing the cows indoors effectively restricted intake, which has been shown to lower milk protein concentration in studies on stress where Jersey cows used to grazing pasture were housed in a barn and fed a restricted diet (Verkerk *et al.* 1999). Cortisol concentrations were measured to monitor stress but there was no increase in circulating concentrations during the experimental period (Table 4-9). There is evidence that for longer studies circulating cortisol concentrations are not a good indicator as cows adapt to the new situation and there is not a cortisol response (Morrow *et al.* 2000).

Milk fat yield was reduced in both the casein infused and control cows over the 4 days of the HEC. However, milk fat concentration was only reduced in the casein infused

cows. It would appear that the decrease in milk fat yield in the control cows was primarily due to the decrease in milk yield caused by the HEC whereas the decrease in milk fat yield in the casein infused cows was due to a decrease in both milk yield and fat concentration.

The changes in milk fat yield and concentration are in agreement with other HEC studies with concentrate fed cows (Griinari *et al.* 1997b, Mackle *et al.* 1999) and goats (Bequette *et al.* 2001). However, in these studies, the primary cause was seen to be a dilution effect due to an increase in milk yield. However, there is also evidence that it was partly caused by a reduction in precursor supply caused by the effects of insulin on adipose tissue and hepatic ketone body production. Insulin inhibits lipolysis and increases fatty acid re-esterification in adipose tissue, reducing fatty acid mobilisation as indicated by the reduction in circulating concentrations of NEFA (by 67%). The reduction of circulating concentrations of NEFA has been demonstrated consistently during HEC studies in lactating ewes (Chapter 3), and dairy cows (McGuire *et al.* 1995a, Griinari *et al.* 1997b, Mackle *et al.* 1999). β-hydroxybutyrate concentrations were also reduced by 39%, which is slightly lower than the 51% decrease seen in the study with lactating ewes. The reduction in the availability of this milk fat precursor could also affect milk fatty acid composition.

Milk fat samples were analysed to detect any compositional changes due to the HEC reducing precursor supply. Only a small number of samples were analysed from the second period of the cross-over, so only the effect of the HEC was tested and the significance level should be treated with caution. In general, the HEC increased the proportion of short and medium chain fatty acids, while decreasing long chain fatty acids. This indicated that the balance of fatty acids in the milk shifted towards fatty acids synthesised *de novo* (particularly medium chain $C_{10:0}$, $C_{12:0}$, $C_{14:0}$) and $C_{16:0}$ as the proportion of long chain (>$C_{18:0}$ and greater) fatty acids decreased. The changes seen in milk fatty acid composition are very similar to those reported by Griinari *et al.* (1997b) in dairy cows and Bequette *et al.* (2001) in goats. This indicates that the HEC is having a similar effect regardless of feed intake and energy balance.

Despite the change in composition of fatty acids, the cows could not support pre-clamp milk fat yields. Actual yields of most fatty acids except for the longer chain *de novo*

($C_{10:0}$ to $C_{14:0}$) decreased. That the longer chain de novo fatty acid yields were maintained suggests the availability of acetate was less affected than the supply of preformed fatty acids. The decrease in the yield of long chain fatty acids would be expected as these are synthesised from dietary lipids or mobilisation of body reserves and the decrease in circulating NEFA concentrations indicates there was a decrease in body mobilisation.

Circulating IGF-I concentrations increased during the HEC and this is consistent with other studies of this type (McGuire *et al.* 1995a, Griinari *et al.* 1997a; Mackle *et al.* 1999, 2000; Bequette *et al.* 2001). As a result of this increase, IGF- 1 has been suggested as being the regulator for milk protein synthesis (McGuire *et al.* 1995a). However, while intra-arterial and intra-mammary infusion of IGF-1 has been demonstrated to increase milk yield in several studies (Prosser *et al.* 1990, Prosser & Davis 1992), it has not in others (Davis *et al.* 1989). There is no detailed understanding of a specific mechanism through which this happens. As IGF-1 is nutritionally regulated (McGuire *et al.* 1995b, Leonard & Block 1997), the increase in circulating concentrations seen in HEC studies may result from high insulin concentrations plus sufficient glucose signalling to the brain that nutrition is abundant, even when intake is reduced as in the cows in this experiment. This would not indicate a direct role for IGF-1 in increasing milk protein synthesis.

Finally, the change in SCC should be mentioned. As reported in the results, there was an increase in SCC that did not result in mastitis. No bacteriology was done on the milk and as a result of this, it is hard to determine what caused the increase. Although there was a small increase in milk sodium concentrations (Table 4-10), there was no overall change in the sodium:potassium ratio which would support the conclusion that the increase in SCC was not caused by mastitis. Another potential cause may have been infection induced by the jugular catheters. However, when the catheters were removed, there was no indication of infection. Consequently, there is no evidence of what caused the increase or what impact it would have had on milk protein synthesis.

4.6 Conclusion

Data produced from pasture-fed cows in this experiment offers no support for the proposal that insulin (under HEC conditions) stimulates milk protein production. While there was no evidence of insulin increasing production directly, there was a change in nutrient partitioning as defined by the change in utilisation of dietary crude protein from milk protein production. However, this enabled the cows to maintain milk yield and yields of components only for 3 of the 4 days of the HEC. The use of this technique was physically demanding and this suggests that the response to the HEC is one that the cows can support for a limited length of time.

4.7 References

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Chapter 5 Insulin and the regulation of amino acid utilisation by the lactating ewe mammary gland.

5.1 Abstract

Twelve lactating ewes housed indoors were allocated to one of two treatment groups (hyperinsulinaemic euglycaemic clamp (HEC) or control) in a randomised block design experiment. Mammary amino acid uptake from plasma and utilisation for milk protein synthesis was measured during the fourth day of the HEC using the arterio-venous concentration difference (A-V) technique. There was no change in milk protein output during the HEC ($P>0.05$). The HEC induced a significant decrease in arterial concentrations of all essential amino acids (EAA) except for histidine. There was no significant change in mammary blood flow. The mammary uptake of some EAA (isoleucine, leucine, methionine and phenylalanine) was significantly reduced by the HEC. Use of a ^{13}C-leucine infusion demonstrated that leucine oxidation in the mammary gland was not altered during the HEC. The data in this experiment demonstrate the mammary gland can adapt to changing precursor supply to maintain production.

5.2 Introduction

The focus of this thesis is on the role of insulin in milk protein production. The primary aims of Chapters 3 and 4 were to determine the milk protein response of pasture-fed lactating ewes and dairy cows under hyperinsulinaemic euglycaemic clamp (HEC) conditions. Use of the HEC alone or the HEC with supplemental protein did not increase milk protein yield or concentration in pasture-fed animals. However, there was a change in the proportional utilisation of dietary protein for milk protein produced. This was interpreted as a change in nutrient partitioning under the conditions imposed by the HEC. The theory of homeorhesis (Bauman & Currie 1980, Bauman 2000) proposes that there is a co-ordinated response between the hormonal and nutritional environment that allows a co-ordinated partitioning of nutrients between tissues during lactation. It appears that this co-ordinated status is altered during the HEC but it is not clear from the previous studies how this apparent increase in protein utilisation was achieved or by what mechanisms.

This experiment was designed to determine how this increase in dietary crude protein utilisation for milk protein production was achieved. As no HEC x protein interaction was demonstrated in chapters 3 and 4, the HEC alone was used to examine the role of insulin in AA uptake and subsequent utilisation for milk protein synthesis in the mammary gland in this experiment. As a result of the decreases of circulating concentrations of AA reported in Chapters 3 and 4, it was hypothesised that the HEC may increase mammary uptake of AA. Additionally, it may increase mammary blood flow, thereby increasing precursor supply to the gland and/or increase the amount of AA available for milk protein synthesis in the mammary gland by decreasing AA oxidation. To determine these relationships, in conjunction with the HEC, the arterio-venous (A-V) concentration difference technique and a labelled AA (^{13}C-leucine) infusion across the mammary gland were used to determine the response of pasture-fed ewes.

The lactating ewe was utilised as a model for lactating ruminants as the previous HEC experiments (Chapters 3 and 4) demonstrated that the response to the HEC in terms of changes in variables such as hormones, metabolites and AA were similar between the lactating ewes and Jersey cows. However, to minimise the effect of some problems that were been identified in the previous experiments (i.e. stage of lactation, exposure to several insulin infusions), rather than a cross-over design, this experiment was run as a single period of infusion, with ewes being blocked on lambing date to ensure that the experiment was run within the first three weeks of lambing.

5.3 Materials and methods

5.3.1 Animals

Twelve 4 and 5 year old, lactating Romney cross ewes were housed indoors in metabolism crates and individually fed fresh perennial ryegrass (*Lolium perenne*)-white clover (*Trifolium repens*) pasture sufficient for *ad libitum* intake during the 3 day pre-experimental and 6 day experimental period. All ewes underwent surgery under general anaesthesia (3 ml Alfaxan (10 mg/ml alphaxalone) and 50 ml breathable halothane (halothane B.P. 1 ml/ml)) around day 70 of gestation. Twelve ewes were fitted with permanent aortic catheters (custom made using the method of Huntington *et al.* (1989)) and 10 ewes were fitted with transit-time blood flow probes (Transonic Systems Inc.,

Ithaca, New York, USA) around the pudic artery. The probe flex and catheter were exteriorised through the skin near the hip bone, where they were placed in a waterproof pouch that had been glued and stitched to the skin. The ewes were housed indoors for 3 weeks after surgery to recover. Post-surgery care included antibiotcs for 3 days and rectal temperatures were monitored as in Chapter 3. Sutures were removed 10 days after surgery and no ewes suffered any complications. Weekly, the aortic catheters were flushed with heparinsed saline (200 IU/ml + 2 ml antibiotics (Bomacillin LA, procaine penicillin 150g/ml and benzathine penicillin 112.5 mg/ml) New Zealand Pharmaceuticals, New Zealand) to maintain patency. The stitching and glue attaching the pouches containing the probe flex and aortic catheter were also checked and replaced when necessary. Finally, the flow probes were checked to ensure they were working.

After recovery from surgery, the ewes were returned to pasture. To protect and maintain the position of the pouch, the ewes wore a fully body stocking made from setonet netting (Setonet, Seaton Healthcare Group PLC, Oldham, UK). The ewes were bought indoors every week to flush the aortic catheter and check the flow probe and pouch. Three days post-lambing the lambs were removed and the ewes moved indoors.

Four jugular catheters (two in each side) and a milk vein catheter (1.2mm x 0.8mm ID single lumen polyvinylchloride tubing, Critchley Electrical Products Pty Ltd, Auburn NSW 2144, Australia) were implanted using the method described in Chapter 3 when the ewes were brought indoors. The 2 catheters in the right jugular vein were used for the simultaneous insulin and glucose infusions of the HEC. In the left jugular vein, 1 catheter was used for monitoring blood glucose and the remaining catheter was used for the ^{13}C-leucine infusion. The catheter in the milk vein was used for sampling venous blood leaving the mammary gland.

The ewes were machine milked twice a day (at 0730 and 1930 h), following the administration of oxytocin (1 IU/ewe, Oxytocin V, Vetpharm (NZ)) via a jugular catheter to stimulate milk letdown.

5.3.2 Experimental Procedure

5.3.2.1 Design

The twelve ewes were allocated to treatment groups (± insulin) in a randomised block design and subjected to a HEC over a 4 day period. Ewes were blocked in groups of 4 (2 ± insulin) according to date of lambing, so that infusions commenced 7-10 days after lambing.

5.3.2.2 Infusions

The HEC was performed as detailed in Chapter 3. Briefly, insulin derived from bovine pancreas (1μg/kg BW $^{0.75}$, Sigma Chemicals, St Louis, MO.) was infused in a sterile filtered 0.5% bovine serum albumin solution (Immuno Chemical Products Ltd, NZ). The blood glucose concentrations of the ewes were monitored using an Advantage Blood Glucose Meter (Boehringer Mannheim Ltd, NZ). This allowed rapid (within 2 min) determination of blood concentrations for adjusting the glucose infusion rate to maintain euglycaemia during the insulin infusion. The glucose infusion (a sterile 30% w/v glucose solution prepared using food grade dextrose monohydrate and autoclaving; Pure Chem Co. Ltd, Thailand) was used to maintain euglycaemia via variable speed pumps.

Control ewes were infused with an equal volume of the BSA solution. On day 4 of the HEC, ^{13}C-leucine was infused via the jugular vein into all the ewes between 2.30 am and approximately 11 am (time of final sample). The ^{13}C-leucine was administered in a primed (47 mg, equivalent to 26.5 min of infusion) constant infusion (0.74 ml/min) of L-[1-^{13}C]leucine (99 AP, MassTrace, Woburn, MA 01801, USA) by a constant infusion Watson Marlow pump.

5.3.3 Sample analysis and Calculations

5.3.3.1 Milk

Milk yields were recorded by weighing and a sample stored at -20°C for further analysis at each milking. Milk composition was determined by near-infrared spectroscopy in transmission mode (Model 6500, NIRsystems Inc, Silver Spring, MD, USA) with PC software by Infrasoft International (version 3.1) that was calibrated for ewe's milk

(detailed in chapter 3). A sample was processed as skim milk, frozen at –85°C and analysed for individual milk proteins using a nephelometric assay (Collin *et al.* 2001). Milk amino acid composition (exclusive of methionine) was determined in freeze-dried skim milk samples, after HCl hydrolysis (AOAC, 1990), using post-column derivatisation with ninhydrin (PCX 3100 Post Column Reaction Module, Pickering Laboratories, Mountain View, Ca 94043, USA) and a Shimadzu LC10Ai HPLC system (Shimadzu Scientific Instruments Ltd., Columbia, MD 21046, USA). Methionine concentrations were determined using the same system, in samples hydrolysed after oxidation with performic acid (AOAC, 1990).

5.3.3.2 Blood

Five min before starting to collect blood samples, the ewes were primed with a 1 ml bolus injection of 1000 IU/ml sodium heparin (New Zealand Pharmaceuticals, Palmerston North, New Zealand). This enabled integrated A (from the aorta artery) and V (from the mammary vein) blood samples to be collected continuously over 45 min. On the day before starting the HEC, collection of 2 samples commenced at 1200 and 1400 h. On day 4 of the HEC, samples were taken over the afternoon feeding period commencing at 1200, 1400 and 1600 h and again during the final feeding period commencing at 0730, 0900 and 1030 h. Concentrations of insulin were determined on arterial samples only. Glucose, β-hydroxybutyrate, triacyglycerol and AA concentrations were determined on A and V samples. Preliminary analysis (on group one samples) showed there were no significant differences in AA concentrations between the two feeding periods, so only the samples from the second period were analysed for groups two and three.

Amino acids concentrations were analysed using two methods: A sample of whole blood (1 ml) was deproteinised by addition of 0.5 ml 30% trichloroacetic acid (TCA), centrifuged (at 3270 g at 4°C for 15 min) and filtered through a 0.45 μm cellulose syringe filter. This filtrate was used to determine cysteine concentrations by a modified automated method using acid ninhydrin (Gaitonde 1967). The remaining blood was centrifuged at 3270 g at 4°C for 15 min and the resulting plasma was harvested and stored at -85°C until analysed. Amino acid concentrations (exclusive of cysteine) were measured on plasma samples that were mixed with 25 μl of 3 mM nor-leucine as an

internal standard and then deproteinised by ultrafiltration (Centrisart, molecular weight cut-off 10,000; Sartorius AG, Gottingen, Germany). The ultrafiltrate was stored at –85°C until analysed by reverse phase HPLC separation of phenylisothiocyanate derivatives on a Waters PicoTag© column and a Shimadzu LC10/A HPLC system using a modified method of Bidlingmeyer *et al.* 1984 (for full method see Appendix B). Amino acids are grouped as essential (EAA) and non-essential (NEAA) as described in Chapter 3.

For determination of the concentration and isotopic enrichment of ^{13}C-leucine and keto isocaproate (KIC), plasma samples were deproteinised with sulphosalicylic acid (SSA). Internal standard (ketocaproic acid, 1 μmol/ml) was added and the sample centrifuged at 13,000 rpm for 5 min. The resulting supernatant was separated into leucine and KIC fractions on SCX (H+) ion exchange columns. Both fractions were then derivatised using the N,O-+-B method of Calder and Smith (1988). This measures the tertiary-butyldimethylsilyl (+BDMS) derivative of leucine (ions of masses 302.2 and 303.2) and the quinoxalinol-t-BDMS derivative of KIC (ions of masses 359.1 and 260.1). Analyses of both fractions were carried out on a Shimadzu GC (Shimadzu GC-17A) in conjunction with a Shimadzu MS (Shimadzu QP5050A). A 1 μl injection of sample was made, with a 1:20 split ratio with helium as the carrier gas. Analysis of the injected sample was made on a DB%-MS column (J&W, I.D. 0.25 mm, film thickness 25 μm, 30m length). Results are presented as moles percent excess (MPE) with respect to pre-infusion natural abundance of ^{13}C in plasma free leucine and KIC. The KIC concentration was determined from the gas chromatographic-mass spectrometric peak areas, corrected to the known addition of the standard.

Plasma insulin concentrations were measured on arterial samples using a double antibody radioimmunoassay (Flux *et al.* 1984). Intra- and inter-assay coefficients of variation were 8.7% and 12.9% respectively. The mean sensitivity was 22.7 pg insulin/ml.

Plasma glucose, NEFA, β-hydroxybutyrate and triacylglyerol concentrations were measured on A and V samples by enzymatic colorimetric methods that have been adapted to a Cobas Fara II autoanalyser (Hoffman-La Roche Ltd, Basal, Switzerland).

Glucose was measured using the method of Trinder (1969). The NEFA method is based on that of McCutcheon & Bauman (1986), using a Wako C test kit (Wako Pure Chemical Industries Ltd, Osaka, Japan) and the intra- and inter- assay coefficients of variation were 4.6% and 2.3% respectively. The β-hydroxybutyrate method was based on that of Williamson & Mellanby (1974), with the intra- and inter- assay coefficients of variation being 4.7% and 2.1% respectively. The triacylglycerol method was based on that of McGowan *et al.* (1983) and Fossati & Prencipe (1988). The intra- and inter-assay coefficients of variation were 2.4% and 2.7% respectively.

Concentrations of acetate, propionate and butyrate were measured in A and V samples. Whole blood (1 ml) was deproteinised using 2 ml of 0.6 M $HClO_4$, followed by centrifugation at 4°C for 15 min at 4000 rpm. The supernatant was neutralised using 300 µl 3 M KOH, and placed on ice to allow the $KClO_4$ to precipitate out. The supernatant was poured off the precipitate and stored at –85°C until analysis by GC. Immediately prior to analysis by GC, the supernatant was acidified by adding H_3P0_4 and centrifuged for 10 min at 10,000 rpm. The resulting supernatant was analysed on a Shimadzu GC with an acidified (TPA) polyethylene glycol (DB-FFAP) column, (J&W Scientific, I.D. 0.53 mm, 30m length). The sample was injected (volume 1µl, split 1:5) in carrier gas (helium, 1 ml/min mixed with air, 400 ml/min) at an initial temperature of 10°C for 5 min. The temperature was increased at a rate of 10°C/min until 250°C at which it was held for 12 min. The VFA were detected by a flame ionisation detector.

Urinary cortisol and creatinine concentrations were determined on samples that were collected directly after the morning milking. Cortisol was measured using a competitive enzyme-immunoassay developed by Munro & Stabenfeldt (1985). The assay was validated for ovine urine by demonstrating: (i) parallelism between serial dilutions of ovine urine and the standard curve; and (ii) recovery of exogenous cortisol added to ovine urine (C.J.Morrow, per. com.). The creatinine concentration of each urine sample (diluted 1:10 in saline) was determined using a Hitachi 717 autoanalyser and the Roche Diagnostics test (Jaffe method; Roche Package insert, 1997). To control for variations in urine volume and concentration, hormone concentrations were corrected for creatinine concentrations. Accordingly, urine corticosteroid concentrations are expressed as ng cortisol/mg creatinine.

5.3.3.3 Feed intake

Ewes were offered approximately 3 kg DM/ewe/day of pasture that was cut daily, stored in a chiller at 4°C until fed and offered every 6 h at 1200, 1800, 0000 and 0600 h, with water available *ad libitum.* Refusals from each feed were collected from the bins and floor, bulked separately, weighed and the bin refusals subsampled for analysis. Dry matter was calculated on both feed offered and refused and daily dry matter intake (DMI) was measured as offered less refused. Daily samples were analysed for crude protein (CP) and energy (ME) by a scanning spectrometer (model 6500, NIRsystems Inc, Silver Spring, MD, USA) with PC software by Infrasoft International (version 3.1) calibrated for pasture (Corson *et al.* 1999).

5.3.3.4 Blood Flow

Blood flow was measured every day during the HEC in 6 ewes (3 from each treatment) with transit-time, ultrasonic blood flow probes (Transonic Systems Inc., Ithaca, NY). The probes give a measurement of volumetric flow, using the principle of transit time. The probe is placed around the vessel and 2 transducers pass ultrasonic signals back and forth, alternatively intersecting the blood flowing in upstream and downstream directions. The flowmeter derives an accurate measurement of the transit-time it took the wave of ultrasound to travel from one transducer to the other. The difference between the intergrated transit-times is a measure of volume flow (www.lintonist.co.uk/transonic.htm). All probes are factory calibrated.

Blood flow measurements were taken during the A and V blood collections. Each measurement was for 2 minutes on alternate ewes, so that over the three 45 minute collection in each blood sampling period, each ewe had 20 2 minutes measurements. These were averaged to calculate daily blood flow.

The probes measure the blood flow for the half of the gland that the vessel they are around supplies. It was assumed that the venous blood sample was representative of blood from both sides of the gland. As a result of this, the blood flow measured by the probes was used for both sides of the gland, after being corrected by a ratio calculated from milk production from each half of the gland over 1 hour.

Blood flow was also calculated for all ewes on day 4 of the HEC using AA A-V concentration difference and the Fick principle. By using the Fick principle, it is accepted that the flow through an organ can be estimated if the inlet (artery) and outlet (vein) concentrations of an indicator (AA in this case) can be measured together with its uptake. The calculation was based on the method of Davis *et al.* 1988, using the equation

$$\text{MBF}\,(\text{l.hr}^{-1}) = \frac{\text{AA output in milk}\,(\text{mg.hr}^{-1})}{\text{A} - \text{V of AA in blood}\,(\text{mg.l}^{-1})}$$

AA output in milk was calculated assuming 84% of ovine milk protein is synthesised in the mammary gland (Davis & Bickerstaff 1978).

5.3.4 Leucine kinetic calculations

The isotopic enrichment (IE) of leucine in any measured pool was calculated as moles percent excess (MPE) using the formula:

$$\text{IE (MPE)} = \frac{R_t - R_0}{1 + (R_t - R_0)}$$

where R is ratio of mass of monitored fragment containing ^{13}C divided by mass of unlabeled molecule. The subscript t indicates enrichment over time t whereas 0 is the enrichment in background (arterial) samples obtained before infusion (Lobley *et al.* 1996).

5.3.5 Whole body calculations

Leucine whole body irreversible loss rate (ILR) was calculated with the following formula using arterial leucine as the precursor pool:

$$\text{ILR}\,(\text{mmol.hr}^{-1}) = \frac{\text{infusate enrichment}}{\text{MPE}_{\text{A leu}} - 1} \bullet \text{infusion } r\text{ate}$$

where infusate enrichment (MPE) is 0.99%, and infusion rate is in mmol/h (Lobley *et al.* 1996).

Leucine partitioning to the mammary gland (gross uptake:whole body flux) was calculated as

$$(L_A \bullet E_{LA} - L_V \bullet E_{LV}) \bullet \frac{MBF}{E_{LA}} \bullet \frac{1}{ILR_{A\,Leu}}$$

where L_A and L_V are arterial and venous concentrations of leucine, and E_{LA} and E_{LV} are their isotopic enrichments (Bequette *et al.* 1996a). ILR_{ALeu} is the whole body ILR calculated using leucine as the precursor pool.

Leucine ILR was converted to leucine lost/day (g/d) by

$$ILR_{A\,Leu} \bullet 24 \bullet \frac{MW_{Leu}}{1000}$$

where MW_{Leu} is the molecular weight of leucine (131.18).

An equivalent protein flux (g protein/day) was calculated using

$$\frac{\text{ILR } (g.d^{-1})}{\%\text{ AA in whole body protein}}$$

where leucine is 66.9 g of leucine/kg of ILR values (Bequette *et al.* 1996b).

5.3.6 Mammary gland calculations

The calculations for leucine kinetics across the mammary gland were as follows:

Net uptakes for leucine and KIC by the mammary gland were calculated as
(arterial concentration - venous concentration) • MBF.

The equations used for net leucine retention, leucine for protein synthesis and leucine oxidation in the mammary gland are from the model used across the sheep hind limb by Harris *et al.* (1992).

Net leucine retention (mmol/h) was calculated as

$$((L_A - L_V) - (K_A - K_V)) \bullet MBF - \left(C_V \bullet E_{C,A} - \frac{C_A \bullet E_{C,V}}{E_{K,V}} \right) \bullet MBF$$

which is the difference between arterial and venous concentrations of leucine corrected for changes in net output of KIC to $C0_2$. Where L and K are the concentrations (mmol/l) of leucine and KIC in plasma and C is the concentration (mmol/l) of CO_2 in the blood. E signifies the enrichment (MPE) above background samples of the

metabolites. $E_{K,V}$ is the enrichment of KIC in the milk vein and was taken as the most representative of the isotopic activity at the site of branch-chain 2-oxo-acid dehydrogenase in the tissues.

Leucine for protein synthesis (mmol/h) was calculated as

$$\frac{((L_A \bullet E_{LA} - L_V \bullet E_{LV}) - (K_A \bullet E_{K,A} - K_V \bullet E_{K,V})) \bullet MBF - (C_V \bullet E_{C,A} - C_A \bullet E_{C,V}) \bullet MBF}{Sy}$$

where Sy is the single most practical substitute for the true precursor. The different pools label to different extents, so values obtained depend on which pool is chosen as the most representative of the true precursor (the appropriate aminoacyl-t-RNA) (Harris *et al.* 1992). In these calculations the isotopic enrichment of arterial leucine (E_{LA}) was used.

Leucine oxidation (LO) across the mammary gland was that isotope lost as CO_2 and was calculated using the equation:

$$LO(mmol.hr^{-1}) = \frac{C_V \bullet E_{C,A} - C_A \bullet E_{C,V}}{E_{K,V}} \bullet MBF$$

Total protein synthesis in the mammary gland was estimated using the equation

$$\frac{((L_A \bullet E_{L,A} - L_V \bullet E_{L,V}) \bullet MBF) + ((K_A \bullet E_{K,A} - K_V \bullet E_{K,V}) - (C_V \bullet E_{C,A} - C_A \bullet E_{C,V}) \bullet MBF)}{E_y}$$

where Ey is either enrichment of leucine or KIC in the milk vein plasma (Bequette *et al.* 1996b).

5.3.7 Statistical analysis

The data generated in this experiment were analysed as a randomised block design. As the ewes were blocked according to lambing date, analyses included HEC as a fixed treatment effect with block as a random effect and time as a repeated factor. Analyses were preformed using the procedure GLM from the statistical package SAS (1988). The model used for the analyses was:

$$Y_{ijkl} = \mu + \delta_k + \pi_{(l)k} + \alpha_i + \beta_j + \varepsilon_{ijkl}$$

where μ is the mean, δ_k is the random effect due to the group k, $\pi_{(l)k}$ is the l^{th} ewe in group k, α_i is the fixed effect due to the treatment i, and ε_{ikl} is the error term. Results

are expressed as least squares means ± standard error of the mean (± SEM). All treatment and period effects and their interactions were tested, and probability values were generated to compare between treatments. Normality of the data was tested by plotting the standardised residuals against the standardised predicted values of the response variables. No plots showed a pattern that would indicate that the normality assumption of the ANOVA model should be questioned and that the data should be transformed.

5.4 Results

5.4.1 Production data

Plasma insulin concentrations were not significantly different between the HEC and control ewes on the pre-infusion day. On day 4 of the HEC, concentrations had significantly increased in the insulin treated ewes compared to the control ewes (399 ± 20 vs 179 ± 20 pg/ml, P>0.001). While the amount of glucose infused to maintain euglycaemia increased from day 1 to day 4 of the HEC (4.9 ± 0.4 vs 5.7 ± 0.4 g/h), this increase was not significant.

Feed intake data are presented in Table 5-1. These data were adjusted with a covariate determined from the average of intakes for the 2 days preceding the start of the HEC. Uncorrected data are presented in Appendix C. There were no significant differences in any intake variables between insulin treated and control ewes on day 4 of the HEC (Table 5-1).

Table 5-1 Comparison of intakes of dry matter, crude protein, energy and energy balance and dietary crude protein (CP) utilisation (LSMeans ± SEM) on day 4 of the hyperinsulinaemic euglycaemic clamp (HEC) between treated (HEC) and control ewes.

	Treatment		
	HEC	Control	± SEM
Intakes			
Dry matter (kg/d)	1.34	1.49	0.08
Crude protein (g/d)	284	292	9
Energy (MJ ME/d)	14.9	16.3	0.9
Total Energy (MJ ME/d)[1]	16.6	16.3	0.9
Energy Balance[2]	-7.7	-8.9	0.9
Efficiency of dietary CP utilisation (g/g)[3]	0.27	0.28	0.02

Treatments: HEC = hyperinsulinaemic euglycaemic clamp (n=6), control = BSA infused group (n=6).
[1] Total energy (MJ ME) intake was calculated by combining dietary intake with additional ME available from the glucose infusion.
[2] Calculated as total energy intake (MJ ME) from diet + casein + glucose – energy output (MJ ME) estimates of maintenance requirements (0.238/kg liveweight) + the energy content of milk produced (4.7 MJ ME/kg).
[3] Efficiency of dietary crude protein (CP) utilisation for milk protein production. Calculated as g milk protein produced per g of CP intake.

Milk yield and yields of individual milk components were significantly greater in the control ewes on the 2 days before the start of the HEC. As there was no change in milk yield in both the insulin treated and control ewes during the HEC, the average of these days for each component was used as a covariate and the adjusted data are presented in Table 5-2. The unadjusted data are presented in Appendix D.

Table 5-2 Comparison of milk yield, yield and concentration of milk components and 4 individual milk proteins (LSMeans ± SEM) on day 4 of the hyperinsulinaemic euglycaemic clamp (HEC) between treated (HEC) and control ewes.

	Treatment		
	HEC	Control	P value
Milk yield (g/d)	1475 (±49)	1523 (±49)	ns
Component yield (g/d)			
Total milk solids [1]	229 (±17)	269 (±17)	ns
Crude protein	75.9 (±3.8)	83.9 (±3.8)	ns
Fat	69.7 (±6.5)	105.0 (±6.4)	0.05
Lactose	66.4 (±4.8)	81.0 (±4.8)	ns
Composition (%)			
Total milk solids	15.1 (±0.4)	17.9 (±0.4)	0.001
Crude protein	5.2 (±0.1)	5.5 (±0.1)	0.01
Fat	4.4 (±0.3)	6.1 (±0.3)	0.001
Lactose	4.5 (±0.1)	5.0 (±0.1)	0.01
Individual milk proteins (g/l)			
β-casein	28.7 (±1.9)	29.0 (±1.9)	ns
β-lactoglobulin	7.3 (±0.6)	6.4 (±0.6)	ns
Immunoglobulins	0.97 (±0.07)	0.79 (±0.07)	ns
κ-casein	4.2 (±0.3)	3.7 (±0.3)	ns

Treatments: HEC = hyperinsulinaemic euglycaemic clamp (n=6), control = BSA infused group (n=6).
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant.
[1] Total weight of milk solids as determined by freeze drying.

There were no significant differences in milk, crude protein or lactose yield between the insulin treated and control ewes on day 4 of the HEC. However, milk fat yield was significantly lower in the insulin treated ewes compared to control ewes, and as a result of this, so was the total solids yield. Concentrations of crude protein, lactose, fat and total milk solids were all significantly lower in insulin treated ewes on day 4 of the HEC.

Milk samples were analysed for four of the individual milk proteins for which the probes used in this method were available. There was no effect of the HEC on the concentration of β-casein (β-CN), β-lactoglobulin (β-LG), κ-casein (κ-CN) or immunoglobulins (IgG) on day 4 (Table 5-2).

Values for arterial concentrations, A-V differences and extraction efficiencies for acetate, triacylglycerols and β-hydroxybutyrate are presented in Table 5-3. On day 4 of the HEC, there was no significant difference in arterial acetate concentration or A-V difference or extraction efficiency between the insulin treated and control ewes. Of the other VFA (propionate and butyrate), it was not possible to determine concentrations on sufficient samples to estimate any changes in concentration.

Table 5-3 Arterial concentrations, A-V differences and mammary gland extraction efficiencies (LSMeans ± SEM) of acetate, triacylglycerols and β-hydroxybutyrate during day 4 of the hyperinsulinaemic euglycaemic clamp (HEC) between treated (HEC) and control ewes.

	Treatment		
	HEC	Control	P value
Arterial Concentration (mM)			
Acetate	2.4 (±0.3)	2.0 (±0.3)	ns
Triacylglycerols	0.15 (±0.01)	0.17 (±0.01)	ns
β-hydroxybutyrate	0.48 (±0.03)	0.72 (±0.03)	0.001
A-V Difference (mM)			
Acetate	1.8 (±0.3)	1.3 (±0.3)	ns
Triacylglycerols	0.003 (±0.01)	0.015 (±0.01)	ns
β-hydroxybutyrate	0.22 (±0.02)	0.37 (±0.02)	0.001
Extraction Efficiency (%)			
Acetate	61 (±4)	52 (±4)	ns
Triacylglycerols	-2 (±8)	5 (±8)	ns
β-hydroxybutyrate	44 (±2)	51 (±2)	0.05

Treatments: HEC = hyperinsulinaemic euglycaemic clamp (n=6), control = BSA infused group (n=6).
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.

There was no difference in arterial plasma concentrations, A-V difference or extraction efficiency for triacylglycerols between the insulin treated and control ewes on day 4 of the HEC. Whereas arterial β-hydroxybutyrate concentrations, A-V differences and extraction efficiency in the insulin infused ewes were significantly lower than the control ewes.

Creatinine and cortisol concentrations were measured in urine samples on each of the 4 days of the HEC. There was no effect of treatment on either creatinine or cortisol

concentrations. On day 4, creatinine concentrations were 0.13 ± 0.03 mg/ml in the insulin treated ewes vs 0.15 ± 0.03 mg/ml in the control ewes. Cortisol concentrations (221 ± 76 ng cortisol /mg creatinine) in the insulin treated ewes were not significantly different from 289 ± 76 ng cortisol /mg creatinine in the control ewes.

5.4.2 Amino acid and mammary blood flow data

Plasma arterial concentrations of EAA and NEAA are presented in Table 5-4. As there were no significant differences between the HEC and control ewes in plasma AA concentrations measured on the pre-infusion day, AA concentrations were not analysed by covariate analysis. With the exception of arginine and histidine, arterial concentrations of all other EAA measured were significantly lower in the insulin treated ewes on day 4 of the HEC than those in the control ewes. The concentration of BCAA decreased by 29 %, and the remaining EAA (less histidine) decreased on average by 18%. Overall, concentrations of NEAA were 9% lower in the HEC treated ewes in comparison to the control ewes. This was due to significantly lower concentrations of aspartate, glutamate and proline in the insulin treated ewes. Despite a numerical decrease, concentrations of serine, asparagine, glutamine, alanine and tyrosine were not significantly different. Glycine was the only AA that had a significantly higher (14%) concentration in the insulin treated ewes.

Table 5-4 Comparison of plasma arterial concentrations of amino acids (LSMeans ± SEM) on day 4 of hyperinsulinaemic euglycaemic clamp (HEC) between treated (HEC) and control ewes.

	Treatment		
	HEC	Control	P value
Essential Amino Acids (μM)			
Arginine	79.6 (±7.1)	94.7 (±7.1)	ns
Cysteine	70.1(±3.5)	82.9 (±3.5)	0.05
Histidine	57.7 (±3.4)	58.4 (±3.4)	ns
Isoleucine	65.6 (±3.5)	97.0 (±3.5)	0.001
Leucine	116.0 (±6.0)	165.4 (±6.0)	0.001
Lysine	75.5 (±5.6)	92.5 (±5.4)	0.05
Methionine	27.3 (±1.4)	34.3 (±1.4)	0.01
Phenylalanine	50.7 (±1.6)	61.9 (±1.6)	0.001
Threonine	104.4 (±4.9)	134.5 (±4.9)	0.001
Tyrosine	70.8 (±2.0)	71.5 (±2.1)	ns
Valine	144.3 (±8.4)	194.3 (±8.1)	0.001
Non Essential Amino Acids (μM)			
Alanine	135.6 (±4.4)	144.0 (±4.8)	ns
Asparagine	61.2 (±2.5)	68.6 (±2.5)	†
Aspartate	6.2 (±0.4)	9.2 (±0.4)	0.001
Glutamine	353.5 (±19.1)	372.1 (±19.6)	ns
Glutamate	87.6 (±3.1)	134.5 (±3.1)	0.001
Glycine	496.5 (±16.5)	435.8 (±16.5)	0.05
Proline	102.5 (±3.5)	116.5 (±3.5)	0.05
Serine	67.7 (±3.2)	77.8 (±3.2)	ns

Treatments: HEC = hyperinsulinaemic euglycaemic clamp (n=6), control = BSA infused group (n=6).
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.

A-V differences across the mammary gland for individual EAA and NEAA were measured on day 4 of the HEC (Table 5-5). In the EAA, the HEC significantly decreased the A-V differences of the BCAA and threonine by 22%. While there tended to be smaller A-V differences in the HEC ewes for cysteine and phenylalanine, there were no significant changes in the A-V difference for histidine, lysine and methionine. This is despite very low A-V differences for cysteine and histidine. The HEC significantly decreased the A-V differences for asparate and glutamate in the NEAA. There were no significant changes in the A-V differences of the remaining NEAA, despite a numerical increase in the A-V difference for alanine.

Table 5-5 Comparison of plasma amino acid A-V differences (LSMeans ± SEM) across the mammary gland on day 4 of the hyperinsulinaemic euglycaemic clamp (HEC) between treated (HEC) and control ewes.

	Day 4 HEC		
	HEC	Control	P value
Essential Amino Acids (μM)			
Arginine	37.8(±5.0)	46.2 (±4.5)	ns
Cysteine	1.8 (±2.6)	8.9 (±2.6)	†
Histidine	6.2 (±4.4)	10.4 (±3.9)	ns
Isoleucine	38.8 (±2.5)	50.4 (±2.2)	0.01
Leucine	71.2 (±3.9)	87.3 (±3.5)	0.01
Lysine	39.8 (±3.4)	48.3 (±3.1)	†
Methionine	17.1 (±1.3)	20.3 (±1.2)	†
Phenylalanine	20.7 (±1.3)	27.3 (±1.3)	0.01
Threonine	28.7 (±3.3)	39.5 (±2.9)	0.05
Tyrosine	23.1 (±2.2)	29.7 (±2.0)	0.05
Valine	65.4 (±3.6)	77.9 (±3.0)	0.05
Non Essential Amino Acids (μM)			
Alanine	34.9 (±3.4)	34.1 (±3.2)	ns
Asparagine	24.8 (±2.9)	27.5 (±2.6)	ns
Aspartate	2.3 (±0.5)	4.3 (±0.4)	0.01
Glutamine	5.5 (±13.9)	33.3(±12.3)	ns
Glutamate	48.6 (±3.8)	70.1 (±3.4)	0.001
Glycine	-8.6 (±17.4)	10.9 (±15.5)	ns
Proline	25.8 (±2.6)	30.0 (±2.3)	ns
Serine	30.4 (±3.4)	40.9 (±2.8)	0.05

Treatments: HEC = hyperinsulinaemic euglycaemic clamp (n=6), control = BSA infused group (n=6).
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.

Mammary extraction efficiency data are presented in Table 5-6. In the EAA, the HEC increased the mammary extraction efficiency of the BCAA by 17% in comparison to the control ewes. This is in contrast to tyrosine, for which the extraction efficiency was 23% lower in the HEC ewes. For the NEAA, only the reduction in the extraction efficiency of serine was significantly different after 4 days of the HEC.

Table 5-6 Comparison of mammary extraction efficiency (%) by the mammary gland of plasma amino acids (LSMeans ± SEM) on day 4 of the hyperinsulinaemic euglycaemic clamp (HEC) between treated (HEC) and control ewes.

	Treatment		
	HEC	Control	P value
Essential Amino Acids (%)			
Arginine	47.4 (±3.9)	51.2 (±3.6)	ns
Cysteine	5.6 (±6.2)	20.3 (±6.2)	ns
Histidine	14.2 (±5.0)	21.0 (±4.5)	ns
Isoleucine	61.0 (±3.1)	51.8 (±2.8)	0.05
Leucine	62.5 (±2.9)	53.9 (±2.6)	0.05
Lysine	53.2 (±3.9)	53.2 (±3.9)	ns
Methionine	58.8 (±3.3)	61.5 (±3.0)	ns
Phenylalanine	39.5 (±2.2)	44.8 (±2.1)	ns
Threonine	29.8 (±2.0)	30.1 (±1.7)	ns
Tyrosine	32.2 (±2.8)	41.7 (±2.5)	0.05
Valine	47.3 (±2.6)	40.1 (±2.2)	†
Non Essential Amino Acids (%)			
Alanine	25.3 (±2.4)	23.6 (±2.2)	ns
Asparagine	40.1 (±2.6)	39.9 (±2.3)	ns
Aspartate	35.3 (±5.0)	46.2 (±4.5)	ns
Glutamine	8.6 (±3.2)	1.3 (±3.7)	ns
Glutamate	55.1 (±3.1)	52.0 (±2.8)	ns
Glycine	-1.6 (±3.2)	1.9 (±2.9)	ns
Proline	27.8 (±2.4)	26.0 (±1.9)	ns
Serine	46.6 (±3.2)	56.7 (±2.5)	0.05

Treatments: HEC = hyperinsulinaemic euglycaemic clamp (n=6), control = BSA infused group (n=6).
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.
Extraction efficiency (%) was calculated as A-V/A x 100.

Estimates of mammary blood flow from transonic blood flow probes were compared with estimates calculated from arterial plasma A-V differences of methionine, and phenylalanine+tyrosine and the Fick principle in the six ewes with functioning flow probes. On day 4 of the HEC there was no significant difference in mammary blood flow between the control and insulin clamp ewes with any of the three methods used, although the estimates using phenylalanine+tyrosine were numerically higher (Table 5-7).

Table 5-7 Comparison of blood flow estimates by different methods with 6 ewes and by methionine concentration with 12 ewes (LSMeans ± SEM) on day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Treatment			
	HEC	Control	± SEM	P value
Blood flow (ml/min)	(n=3)	(n=3)		
Comparison of different methods of blood flow estimation				
Transonic flow probe	714	712	167	ns
Methionine	743	713	167	ns
Phenylalanine+Tyrosine	1007	810	167	ns
Mammary blood flow estimates using methionine concentrations				
	(n=6)	(n=6)		
	633 (±51)	692 (±48)		ns

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, control = BSA infused group.
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.

However, blood flow estimates were required for the amino acid uptake and leucine kinetic calculation for each individual ewe. The ANOVA showed no significant difference between the two methods using amino acid concentrations but there was a large error. To determine which amino acid concentration method should be used to estimate blood flow, a linear regression was conducted to see if there was a significant relationship between the mammary blood flow measured by flow probe and the blood flow calculated with AA A-V concentration difference and the Fick principle (Fig. 5-1).

Based on the similarity of blood flow values predicted by the transonic flow probe and methionine estimate from the ANOVA, and the stronger regression relationship, it was decided to calculate individual blood flows using methionine A-V concentration difference and the Fick principle. For the 12 ewes in the experiment on day 4 of the HEC, blood flow was not significantly different (Table 5-7) between treated and control ewes. The ratio of mammary blood flow:milk yield in the insulin treated group was 562 ± 39 versus 605 ± 34 in the control ewes and again these were not significantly different. Ratio estimates on day four of the HEC in the insulin treated group ranged from 568 to 761, whereas in the control ewes, estimates ranged from 425 to 788.

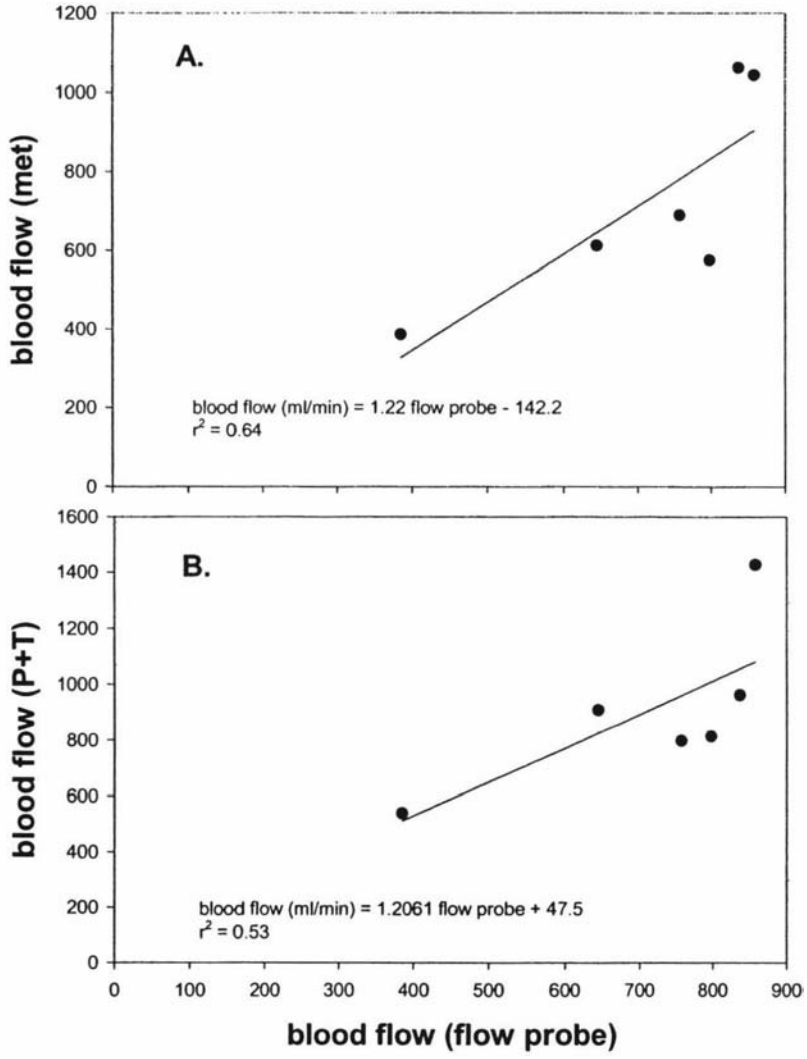

Fig. 5-1 The relationship between blood flow measured with the transit time flow probes (ml/min) and amino acid A-V differences using the Fick Principle. A) shows the linear regression of flow probe against estimates based on methionine A-V differences, whereas B) shows the linear regression of flow probe against estimates based on the average of phenylalalnine and tyrosine (P+T) A-V differences.

Individual AA uptakes by the mammary gland are presented in Table 5-8. Amino acid uptakes were calculated using the estimates of blood flow based on methionine concentrations for the 12 ewes (Table 5-7). For the EAA, uptakes were lower for the BCAA, methionine and phenylalanine in the HEC ewes. There were no significant differences in uptakes of the NEAA except for glutamate, which was significantly lower in the HEC ewes.

Table 5-8 Comparison of net uptakes (mM/h) of plasma amino acids (LSMeans ± SEM) by the mammary gland on day 4 of the hyperinsulinaemic euglycaemic clamp (HEC) between treated (HEC) and control ewes.

	Treatment		
	HEC	Control	P value
Essential Amino Acids (mM/h)			
Arginine	1.78 (±0.13)	1.93 (±0.10)	ns
Histidine	0.57 (±0.22)	0.55 (±0.17)	ns
Isoleucine	1.71 (±0.12)	2.05 (±0.09)	0.05
Leucine	2.91 (±0.16)	3.62 (±0.13)	0.01
Lysine	1.82 (±0.11)	1.89 (±0.08)	ns
Methionine	0.67 (±0.03)	0.84 (±0.02)	0.001
Phenylalanine	0.84 (±0.04)	1.09 (±0.04)	0.001
Threonine	1.45 (±0.18)	1.73 (±0.14)	ns
Tyrosine	0.89 (±0.22)	1.31 (±0.17)	ns
Valine	2.67 (±0.19)	3.13 (±0.15)	†
Non Essential Amino Acids (mM/h)			
Alanine	1.65 (±0.18)	1.40 (±0.15)	ns
Aspartate	0.13 (±0.02)	0.14 (±0.02)	ns
Glutamate	2.01 (±0.18)	2.70 (±0.14)	0.05
Glycine	1.13 (±0.54)	0.66 (±0.42)	ns
Proline	1.31 (±0.14)	1.25 (±0.11)	ns
Serine	1.44 (±0.14)	1.56 (±0.11)	ns

Treatments: HEC = hyperinsulinaemic euglycaemic clamp (n=5), control = BSA infused group (n=6).
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.

A ratio of mammary uptake of AA:AA secreted in milk protein was calculated by division of A-V differences for individual AA with their content in milk protein (g/100g). Individual ratios are then expressed relative to methionine. There were no significant differences in the ratios of AA uptake:AA secreted in milk protein in either EAA or NEAA (except for glutamate) between the HEC and control ewes (Table 5-9). There was a deficit in uptake in both the insulin clamp and control ewes in histidine,

phenylalanine, and lysine. For the NEAA, all were deficient in uptake relative to output in the HEC and control ewes.

Table 5-9 Ratio of mammary AA uptake:AA secreted in milk protein during day 4 of the hyperinsulinaemic euglycaemic clamp (LSMeans ± SEM) between treated (HEC) and control ewes.

	Treatment		
	HEC	Control	P value
Essential Amino Acids			
Arginine	1.79 (±0.15)	1.86 (±0.14)	ns
Histidine	0.30 (±0.18)	0.41 (±0.7)	ns
Isoleucine	0.93 (±0.05)	0.98 (±0.05)	ns
Leucine	0.83 (±0.04)	0.83 (±0.04)	ns
Lysine	0.60 (±0.05)	0.58 (±0.04)	ns
Methionine	0.90 (±0.06)	1.17 (±0.05)	ns
Phenylalanine	0.65 (±0.04)	0.68 (±0.04)	ns
Threonine	1.21 (±0.09)	1.19 (±0.08)	ns
Tyrosine	0.79 (±0.06)	0.79 (±0.06)	ns
Valine	1.19 (±0.06)	1.15 (±0.05)	ns
Non Essential Amino Acids			
Alanine	0.60 (±0.05)	0.58 (±0.04)	ns
Aspartate	0.03 (±0.01)	0.05 (±0.01)	ns
Glutamate	0.28 (±0.02)	0.33 (±0.02)	0.05
Glycine	0.50 (±0.29)	0.21 (±0.25)	ns
Proline	0.30 (±0.03)	0.24 (±0.02)	ns
Serine	0.48 (±0.05)	0.52 (±0.04)	ns

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, control = BSA infused group.
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.
Ratio was calculated as A-V of AA/proportion in milk protein (Davis *et al.* 1978).

There were no significant differences in arterial plasma glucose concentrations between the HEC ewes and control ewes, although the A-V difference and extraction efficiency were lower in the HEC ewes (Table 5-10). The HEC did not significantly change glucose uptake or lactose output and as a result, there was no difference in the ratio of glucose uptake:lactose output (Table 5-10).

Table 5-10 Comparison of mammary glucose utilisation (LSMeans ± SEM) on day 4 of the hyperinsulinaemic euglycaemic clamp (HEC).

	Day 4 HEC		
	HEC	Control	P value
Arterial glucose (mM)	3.5 (±0.1)	3.5 (±0.1)	ns
A-V difference (mM)	0.81 (±0.40)	0.95 (±0.40)	0.05
Extraction efficiency (%)	23 (±1)	27 (±1)	0.05
Glucose uptake (mg/h)	4.22 (±0.36)	4.74 (±0.36)	ns
Lactose output (mg/h)	2.69 (±0.26)	3.44 (±0.26)	ns
Glucose uptake:lactose output	1.41 (±0.17)	1.61 (±0.17)	ns

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, control = BSA infused group.
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.

5.4.3 Leucine kinetic results

On day 4 of the HEC, arterial concentrations of leucine and KIC were significantly lower for insulin treated ewes compared to control ewes (Table 5-11). The IE of arterial leucine was significantly higher in the HEC ewes, whereas there was no difference in IE for KIC and CO_2.

Whole body leucine ILR was calculated with arterial leucine as the precursor pool (Table 5-11). There was no difference in IRL between the HEC and control ewes. The whole body ILR was converted to the amount of leucine used (g/d) and an equivalent daily protein flux. Both variables tended ($P=0.08$) to be lower in the HEC ewes compared to control ewes. However, there was no significant difference in the amount of leucine partitioned to the mammary gland between the two groups (see Table 5-11).

Table 5-11 Comparison of isotopic enrichments and whole body leucine kinetics (LSMeans ± SEM) on day 4 of the hyperinsulinaemic euglycaemic clamp (HEC) between treated (HEC) and control ewes.

	Treatment		
	HEC	Control	P value
Arterial plasma			
Leucine (μM)	126.0 (±4.3)	156.4 (±4.3)	0.01
IE of Leucine (MPE)[1]	7.62 (±0.20)	6.53 (±0.20)	0.001
KIC (μM)[2]	6.89 (±0.47)	12.52 (±0.47)	0.001
IE of KIC (MPE)	5.45 (±0.01)	5.22 (±0.01)	ns
CO_2 (mmol)	1.16 (±0.05)	1.23 (±0.05)	ns
IE of CO_2 (MPE)	0.0085 (±0.0004)	0.0084 (±0.0004)	ns
Irreversible loss rate of leucine	10.46 (±0.16)	11.21 (±0.16)	0.08
ILR as g leucine/day[3]	29.7 (±1.3)	35.4 (±1.1)	0.08
Daily protein flux (g/d)	444 (±19)	529 (±16)	0.08
Partitioning of leucine to mammary gland[1]	0.32 (±0.01)	0.30 (±0.01)	ns

Treatments: HEC = hyperinsulinaemic euglycaemic clamp (n=6), control = BSA infused group (n=6). LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.
[1] n=5 for HEC treatment.
[1] = IE = isotopic enrichment, MPE = moles percent excess, [2] = KIC = keto isocaproate, 3 = irreversible loss rate converted to grams of leucine/day.

The values from mammary gland leucine kinetic calculations are shown in Table 5-12. Leucine and KIC A-V differences across the mammary gland, net uptakes of leucine and KIC by the mammary gland and the amount of leucine retained by the mammary gland were significantly lower in the HEC ewes. There were no significant differences between the HEC and control ewes for the amount of leucine used for mammary protein synthesis, leucine from intramammary degradation or leucine oxidation. Total gland protein synthesis was also significantly lower (with both precursor pools) in the HEC ewes.

Table 5-12 Mammary gland leucine kinetics (LSMeans ± SEM) in insulin treated and control ewes on day four of the hyperinsulinaemic euglycaemic clamp (HEC).

	Treatment		
	HEC (n=5)	Control (n=6)	P value
A-V difference			
Leucine (μmol/l)	70.2 (±4.2)	89.2 (±4.0)	0.01
KIC (μmol/l)	2.15 (±0.41)	4.92 (±0.41)	0.001
CO_2 (mmol/l)	-0.09 (±0.04)	-0.01 (±0.04)	ns
Leucine uptake (mmol/h)	2.87 (±0.26)	3.57 (±0.13)	0.01
KIC uptake (mmol/h)	0.10 (±0.02)	0.20 (±0.01)	0.001
Leucine retained by MG (mmol/h)	2.73 (±0.16)	3.33 (±0.13)	0.05
Leucine for PS (mmol/h)			
E_{LA}	1.93 (±0.37)	2.29 (±0.31)	ns
Leucine secreted in milk protein (mmol/hr)	2.16 (±0.26)	2.95 (±0.26)	ns
Leucine oxidation (mmol/h)	1.48 (±0.43)	1.54 (±0.35)	ns
Gland protein synthesis (mmol/h)			
E_{LV}	3.33 (±0.16)	4.17 (±0.13)	0.001
E_{KV}	4.11 (±0.19)	4.69 (±0.15)	0.05

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, control = BSA infused group.
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant at $P>0.1$.

5.5 Discussion

This experiment is the third in a programme that was to determine the effect of insulin on milk protein regulation in pasture-fed ruminants, utilising the HEC technique. However, in this experiment the design was altered to more closely examine areas that insulin may influence that had been identified from the previous 2 experiments and to try to avoid some of the confounding factors that had also been identified. Therefore, in this experiment, the HEC technique was used in conjunction with the A-V concentration difference technique and a leucine tracer infusion across the mammary gland of lactating ewes to determine a) production responses to the HEC without the confounding effects such as stage of lactation, reduced DMI or protein supplementation that were seen in the previous chapters, and b) examine AA uptake and subsequent utilisation for milk protein production under HEC conditions.

5.5.1 Milk production data

Use of HEC technique did not result in a milk protein response in pasture-fed, lactating ewes (Table 5-2). This is consistent with previous experiments (Chapters 3 and 4) in pasture-fed ewes and cows, and concentrate-fed cows (Annen *et al.* 1998, Griinari *et al.* 1997). However, it is in contrast to the studies in 'well-fed' concentrate fed cows (McGuire *et al.* 1995, Mackle *et al.* 2000) and goats (Bequette *et al.* 2001) where there was a significant increase in milk protein output in response to the HEC alone.

The insulin infusion doubled arterial insulin concentrations whereas in the previous ewe study (Chapter 3), circulating concentrations increased three fold with the same rate of infusion. However, Chapter 3 used a cross-over design, and if the increase in insulin concentration from the first HEC is considered (Fig. 3-1), there was only a doubling of insulin concentration. This is in contrast to the studies in 'well-fed' concentrate fed cows (McGuire *et al.* 1995, Griinari *et al.* 1997a Mackle *et al.* 2000) and goats (Bequette *et al.* 2001) where the range of increases in insulin concentrations with similar rates of infusion on a body weight basis were 3.3 – 4 fold. These lower insulin concentrations may reflect a higher insulin clearance in early lactation. In both this study, and that described in Chapter 3, the ewes were between 10 – 20 *post partum*, whereas the cows (McGuire *et al.* 1995, Griinari *et al.* 1997, Mackle *et al.* 2000) and goats (Bequette *et al.* 2001) were in mid-lactation (range 147 ± 20 d – 220 ± 11 d *post partum*).

The size of the increase in insulin concentrations may affect the magnitude of responses to the HEC in parameters such as DMI and AA utilisation. With the lower insulin concentrations, there was a small non-significant increase in exogenous glucose use (as measured by the glucose infusion rate) over the 4 days of the HEC, and very little effect on DMI (Table 5-1). While insulin and glucose decrease feed intake by activating satiety centres in the brains of lactating ewes (Deetz & Wangsness 1980, 1981), the data from the second period of HEC in Chapter 3 indicates this is more pronounced when there is a larger increase in insulin concentrations and glucose use. As a result of feed intake and hence CP intake not changing in this study (Table 5-1), there was no change

in the efficiency of dietary crude protein utilisation for milk protein production (Table 5-1), which was in contrast to results reported in Chapters 3 and 4.

Milk fat yield and concentration were significantly reduced in the HEC ewes compared to control ewes (Table 5-2). The increase in insulin concentration was sufficient to reduce arterial concentration of β-hydroxybutyrate but not acetate in the HEC ewes as would be expected if the HEC was affecting ketone body production as demonstrated by Brockman (1990). Although milk fatty acid composition was not determined, the uptake of acetate and β-hydroxybutyrate has been shown to account for 81% of short and medium-chain fatty acids synthesised *de novo* by the ewe mammary gland (King *et al.* 1985). In addition, although β-hydroxybutyrate is considered as a relatively minor milk fat precursor, it may initiate approximately half of the C4 to C12 fatty acids synthesised in the mammary gland (Davis and Collier 1985). Thererefore, a decrease in supply of β-hydroxybutyrate would be expected to affect milk fat composition and yield.

Changes in NEFA concentrations in HEC studies have been interpreted to indicate changes in adipose tissue mobilisation, and the reduced concentrations measured have indicated a reduction in mobilisation of adipose tissue. NEFA concentrations were not measured in this study because of using heparin as the anticoagulant for blood collections but based on the results of Chapter 3, it would also be expected that the HEC would decrease the availability of long chain fatty acids released during adipose tissue mobilisation.

It must be acknowledged that the use of heparin may have compromised the milk fat data presented in this chapter. Heparin is known to remove the enzyme lipoprotein lipase from the capillary endothelium, which prevents the hydrolysis and uptake of blood triglyercides by the mammary gland (Azzara & Dimick 1989). This may be the reason why there is no mammary extraction of triacylglycerols (Table 5-3) in this experiment. However, the data shows no discernable pattern of lower milk fat concentration or yield after administration of heparin for the blood collections. It may be that heparin reduced lipoprotein lipase activity for the duration of the blood collections but that the effect was transient. Heparin administration to lactating goats did not compromise milk fat production as there was not difference in milk volume, fat

concentration or fatty acid composition (McCarthy & Coccodrilli 1975). Overall, it would appear that the changes in precursor supply caused by the HEC are the most likely cause of the reduction in milk fat.

5.5.2 Blood flow

Mammary blood flow is an important determinant of precursor supply to the lactating gland because if blood flow is altered, the supply of precursors may also be changed. This is demonstrated in the high correlation shown between mammary blood flow and milk yield (Linzell 1974). While extraction efficiency of precursors can be estimated by A-V concentration difference across the mammary gland, this must be combined with an estimate of blood flow to the mammary gland to estimate uptake (mass per unit time) of metabolites over a given period (Linzell 1974).

There was no increase in mammary blood flow in response to the HEC (Table 5-7), which is in contrast to Mackle *et al.* (2000) and Bequette *et al.* (2001), where mammary blood flow increased by 42%. However, in these latter studies there was a galactopoietic effect of the HEC and glucose uptake increased at a similar rate to blood flow. In addition, Bequette *et al.* (2001) reported that the increase in blood flow paralleled the increase in milk yield and milk protein yield, whereas the increase in milk protein yield was proportionally more in Mackle *et al.* (2000). It appears under HEC conditions that the galactopoietic effect and increase in blood flow are related, although what regulates this is not known. Prosser *et al.* (1996) reviewed a number of locally produced vasoactive compounds in the mammary micro vasculature that potentially regulate mammary blood flow. These provide the mechanisms by which the mammary gland can control its own blood supply and thereby regulate precursor supply for milk synthesis. Support for this concept of local regulation was demonstrated by Bequette *et al.* (2000) who found that goats fed a histidine-deficient diet increased mammary blood flow (by 50%) and mammary histidine extraction rates (from 17-90%) to compensate for the reduction in histidine availability. However, what signals, triggers or regulates these compounds are not known.

Although there was no increase in mammary blood flow, glucose uptake:lactose output ratio and glucose extraction in this experiment, the values are similar to experiments by

Davis *et al.* (1978); Pethick & Lindsay (1982); Fleet & Mepham (1985). While the ratio of blood flow:milk yield (range 562:1 – 605:1, Chapter 5) is lower than 870:1 (Davis *et al.* 1978), this may be due to several reasons. That there may have been a contribution of sampling or analytical error must be acknowledged. However, in the study by Davis *et al.* (1978) the ratio of 870:1 was calculated over the whole lactation (up to 71 days *post-partum*) whereas in our study, it was determined on a smaller number of ewes between 10-17 days *post-partum.* Alternatively it may be because of the choice of methionine concentration to estimate blood flow.

As shown in Table 5-7, mammary blood flow in this experiment was measured directly using transit time flow probes and this was compared to indirect estimation by the Fick principle using AA A-V concentration differences of methionine and phenylalanine+tyrosine. There is considerable debate over the accuracy of different methods of measuring mammary blood flow and the data collected in this experiment allowed a comparison of several methods.

The data in this experiment were first analysed with ANOVA, which showed no significant difference between mammary blood flow estimates obtained by different methods. This is supported by Davis *et al.* (1988) where no difference was found between estimates of mammary blood flow using probes or phenylalanine+tyrosine. Therefore a linear regression was used to determine the relationship between the AA methods and the transit time flow probe (Fig 5.1). Numerically, the flow probe measurement and methionine estimate were similar and the methionine estimate was used for all calculations where estimates of blood flow were required.

Using transit-time flow probes provides an independent blood flow measurement as opposed to an estimation of blood flow by using the AA method. However, there are also problems associated with using the probes include correct placement of the probe around pudic artery (Gorewit *et al.* 1989), as if incorrectly placed, the probes can underestimate flow. Farr *et al.* (2000) reported that in lactating goats, the mammary blood flow estimated by the indicator-dilution technique was 1.5 times larger than that recorded by the transit time probes. In addition, probes can malfunction due to the early placement during gestation. In this experiment, the probes were fitted around day 70 of gestation to prevent harm to the fetus or abortion occuring as a result of the general

anaesthesia. Of the 10 ewes that were fitted with transonic flow probes, only 6 provided usable data. While 1 ewe was not used in the experiment because of mastitis, there was incorrect placement around the pudic artery in another ewe and two further probes malfunctioned.

However, the use of AA concentration and the Fick principle also has complications. This method relies on the assumptions that there is a 1:1 uptake:output by mammary gland of the AA being used, and that these AA, (for example, either methionine or phenylalanine+tyrosine) are not metabolised by the mammary gland. If there is an alternative source such as erythrocytes or peptides contributing to mammary uptake of the AA being used, mammary blood flow would be over-estimated (Hannigan *et al.* 1991).

Although there may be some disagreement with using plasma methionine concentrations to estimate blood flow (e.g. Pethick & Lindsay 1985), other studies have shown that there is considerable contribution of erythrocytes and peptides to the supply of methionine, lysine, phenylalanine and tyrosine for milk protein output (Bequette *et al.* 1999, Pacheco-Rios *et al.* 1999). Therefore, in this study, it was decided that because of the similarity in values between the probe and methionine concentrations, the methionine method was the best one to use.

5.5.3 Amino acid utilisation by the mammary gland

The A-V concentration difference technique was used to determine if AA utilisation by the mammary gland was influenced by higher insulin concentrations during the HEC. The decrease in arterial concentrations of most EAA during the HEC is consistent with changes in arterial concentrations seen in studies by Mackle *et al.* (2000) in concentrate fed cows and Bequette *et al.* (2001) in goats. There is a difference in the severity of the reduction in arterial concentrations, with concentrations of BCAA in this study decreasing on average by 29% compared to 55% in the studies by Mackle *et al.* (2000) and Bequette *et al.* (2001). The remaining EAA decreased by 18%, which is similar to the 23% reduction reported by Mackle *et al.* (2000). The magnitude of effect may be dependent on the absolute change in insulin concentrations i.e. in our study the decrease

in arterial AA concentrations may have been less because of the smaller increase in insulin concentration.

Although the increase in insulin concentrations during the HEC was not as large, the response in terms of changes in A-V differences, extraction efficiencies and uptakes were similar to those reported by Mackle *et al.* (2000). However, in this study, the HEC ewes showed an increased extraction efficiency of the BCAA by 17%. This is in contrast to the results of Mackle *et al.* (2000), where there was a greater increase in extraction of BCAA (40%), arginine and lysine (20%).

An increase in mammary gland uptake of AA and/or blood flow are two ways that AA supply for milk protein synthesis can be maintained or increased. In this study with lactating ewes the HEC caused a reduction in the uptake of isoleucine, leucine, methionine, phenylalanine and valine and there was no increase in mammary blood flow to compensate for this. This is in contrast to the study of Bequette *et al.* (2001), where, although the HEC reduced uptakes of histidine, isoleucine, leucine, valine lysine and threonine and several NEAA, mammary blood flow increased by 42%.

As there was no significant change in mammary blood flow from the HEC, a ratio of AA uptake relative to output in milk protein was calculated by dividing A-V differences for individual AA by their content in milk protein (g/100g) (see Davis *et al.* 1978). Individual ratios are expressed relative to methionine, although in the control ewes the ratio is high, given that as a Group 1 amino acid it would be expected to be 1:1. The analysis shows insufficient uptake of histidine, isoleucine, leucine, lysine, phenylalanine and tyrosine to account for their output in milk protein.

It is generally accepted that EAA such as arginine and the BCAA are taken up in excess of their output by the mammary gland. Therefore, the insufficient uptake of isoleucine and leucine in this analysis suggests that use of methionine may be underestimating the contribution of the plasma free AA to milk protein output. As methionine A-V differences were used to estimate mammary blood flow, this suggests blood flow was underestimated in this experiment.

However, some of the deficit in the uptake of plasma free AA for milk protein may be reduced by alternative sources of AA such as erythrocytes and peptides. There is evidence of AA supply from these sources in studies by Bequette *et al.* (1999), Pacheco-Rios *et al.* (1999), Mabjeesh *et al.* (2000). Pacheco-Rios *et al.* (1999) demonstrated in pasture-fed cows AA uptake from plasma was insufficient for milk protein output of histidine, lysine, phenylalanine and tyrosine. However, when whole blood was analysed in that study, the uptakes of lysine, phenylalanine and tyrosine were sufficient to account for the output of these AA in milk protein and the contribution of erythrocytes was seen to be between 5-15%. For histidine, there was a deficit when measured in either plasma or whole blood and it appears that for this AA the plasma free pool may not be the main precursor pool. There may also be a greater dependency on another alternative source such as blood borne peptides as in lactating goats, Bequette *et al.* (1999) showed that between 5-25% of the supply of lysine, methionine, phenylalanine and tyrosine for casein synthesis came from vascular peptides. These may be mechanisms that support milk protein output when precursor supply is reduced, such as early lactation or restricted feed supply.

5.5.4 Leucine kinetics

A ^{13}C-leucine tracer infusion was used to examine if the HEC altered whole body and mammary leucine utilisation, particularly oxidation. The ^{13}C-leucine was infused during the final day of HEC. Leucine was chosen to examine whole body and mammary utilisation because of the large decrease in circulating concentrations seen during the HEC (Chapters 3 and 4) and the metabolism of BCAA is regulated by insulin.

The metabolism of BCAA in mammary cells is similar to that in other tissues, where they are catabolised to yield oxo and iso acids, propionate, acetate, citrate, carbon skeletons for NEAA synthesis (glutamate and aspartate), CO_2 and energy (Wohlt *et al.* 1977). In addition, BCAA are taken up in excess of their output in milk protein (reviewed by Bequette *et al.* 1998). The rate-limiting step of this catabolism pathway is the decarboxylation of the respective keto acids, which is catalysed by the enzyme branched chain keto acid dehydrogenase. The activity of this enzyme is dependent on its phosphorylation state, which insulin regulates. When insulin concentrations are

high, or the concentrations of BCAA are low, the enzyme is inactive and catabolism is inhibited (Block *et al.*1987).

5.5.4.1 Whole body leucine metabolism

It appears that insulin altered whole body leucine utilisation in the HEC ewes by altering this catabolic pathway. The HEC ewes had significantly lower arterial plasma concentrations of leucine and KIC (Table 5-11). While arterial concentrations of leucine were 20% lower in the insulin treated ewes, KIC concentrations were 48% lower. This indicates that the raised insulin concentrations during the HEC inactivated the keto acid dehydrogenase enzyme, reducing the amount of leucine that was deaminated to KIC. However, there was no significant difference in the amount of $C0_2$ measured, which would suggest there was no change in oxidation.

Whole body ILR tended (P=0.08, Table 5-11) to be lower in the HEC ewes. The ILR measures the amount of leucine that is used by the animal (protein synthesis + oxidation). This was converted into the amount of leucine that was used on a g/d basis. The equivalent daily protein flux is the protein equivalent of the ILR and is a minimum estimate of whole body protein synthesis derived from the ILR using a mean value of the leucine content of body protein (66.9 g/kg in sheep, MacRae *et al.* 1993). This flux overestimates protein synthesis as it assumes that the only pathway for leucine utilisation is as a precursor for protein synthesis and does not take into account the proportion of AA used in other processes such as oxidation (Champredon *et al.* 1990). However, despite this, it can be used to compare animals in different physiological states. In this experiment, the equivalent daily protein flux was lower, and as milk protein production was not significantly lower, it may have been that protein tissue degradation was reduced. This reduction would have contributed to the difference seen in the ILR and daily protein flux.

The whole body ILR data presented in Table 5-11 were calculated with arterial leucine enrichment as the precursor pool. There is debate over which precursor pool should be used in these types of calculations (see Harris *et al.* 1992, Tesseraud *et al.* 1993). Briefly, the arterial leucine enrichment was used as it was thought to best represent the primary precursor pool. Leucine enrichment is thought to be diluted as a result of

intracellular degradation whereas KIC is formed intracellularly and is taken to be derived from a pool similar to that used for protein synthesis. However, the KIC pool may be diluted because of by-pass material and as a result of not all cells producing the keto acid (Harris *et al.* 1992).

Insulin did not appear to influence the proportion of the whole-body leucine flux that was partitioned to the mammary gland. The values calculated in this experiment (Table 5-11) are similar to those reported in lactating dairy cows (0.40, 0.42, Bequette *et al.* 1996a) and 0.20 – 0.30 in lactating goats (Bequette *et al.* 1997).

5.5.4.2 Mammary gland leucine kinetics

Milk protein production was maintained in the lactating ewes in chapter 3, where there was a reduction in dietary crude protein available for milk protein synthesis. It was hypothesised that insulin could have reduced AA oxidation, which would enable milk protein output to be maintained. Many amino acids that are taken up in excess of requirements by the mammary gland have been shown to be catabolised. Although there are many functions that the catabolism of AA fulfils, it has not been established whether catabolism occurs in response to excess uptake of AA (passive response) or because the oxidation process is a required event (regulated response) in milk protein synthesis.

Leucine uptake by the mammary gland was not limiting for milk protein synthesis. Although arterial leucine concentrations in the HEC ewes were reduced, net uptakes were greater than output in milk protein in both HEC and control ewes (Table 5-12). There was no difference in leucine oxidation between insulin treated and control ewes (Table 5-12). A schematic model was developed (Fig 5-2), using fluxes values presented in Table 5.12. Fluxes that were not measured were determined by difference. The purpose of this model was to try to determine more information on the fate of leucine in the mammary gland than was provided by the kinetic equations alone.

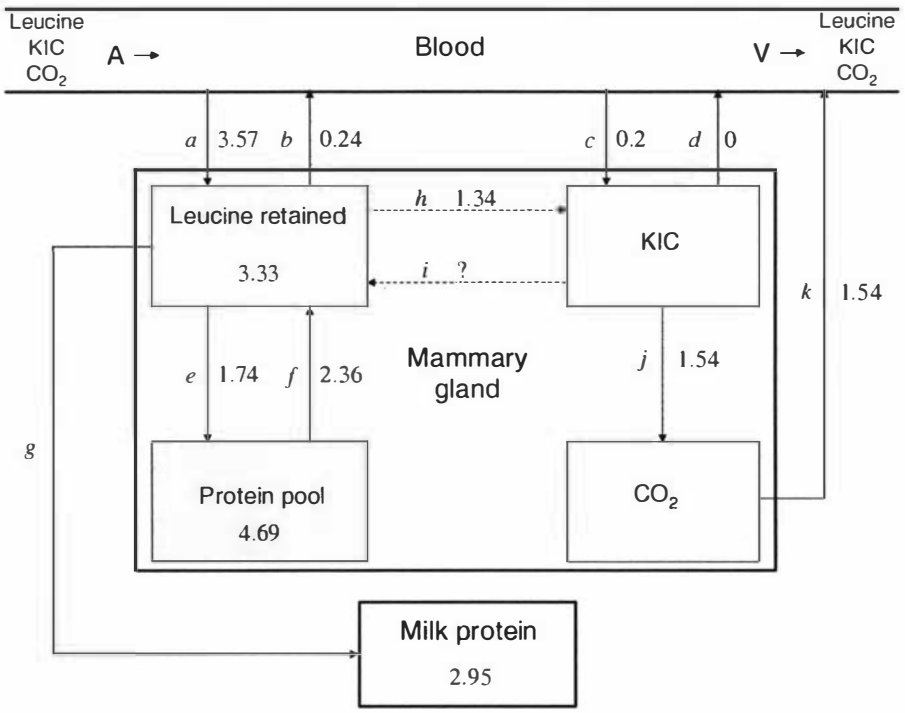

Fig. 5-2 Schematic model showing leucine fluxes in ewe mammary gland of control ewes. Values for solid arrows were measured, while those from dashed arrows were determined by the difference. Symbols: A = arterial, V = venous, KIC = keto isocaproate,

a = uptake of leucine,

b = uptake of leucine – leucine retained,

c = uptake of KIC,

d = net uptake of KIC – KIC retained: not determined and assumed to be 0,

e = what is entering protein pool in the gland by difference of gland protein synthesis – milk protein output,

f = what is leaving protein pool in the gland - assuming steady state is by difference of e + (net uptake of leucine - leucine secreted in milk protein)

g = leucine output in milk protein,

h = leucine being deaminated to KIC, calculated by difference of leucine oxidised less KIC uptake

i = KIC transaminated back to leucine – not determined,

j = KIC to CO_2, and

k = CO_2 released into milk vein blood supply.

The values presented in the figure are for the control ewes. Shaded boxes indicate values for control ewes that are significantly different from HEC ewes. For values, refer to Table 5-12. Flux *f* estimates leucine being released from the tissue protein pool to the leucine pool. It was calculated by difference that an extra 0.62 mmol/h was required from degradation of this pool. This equates to approximately 13g of cellular protein/d being degraded. Assuming a 1.5 kg mammary gland in a lactating ewe with a 300g protein pool, this is clearly unsustainable. If the calculations of mammary blood flow based on mammary methionine uptake were underestimated, this would mean that the leucine uptakes measured were also underestimates. A 20% underestimate of leucine uptake would be sufficient to account for the apparent degradation of the tissue protein pool.

The most important result is that leucine oxidation was not reduced in the HEC ewes and this is in contrast to the study by Bequette *et al.* (2001) where it was reported that oxidation was reduced by 40%. Possible reasons for the difference in oxidation between the ewes in this study and the goats in the previous study are stage of lactation and energy balance. Leucine can be oxidised to CO_2, which provides a small amount of energy. As all the ewes were in negative energy balance, perhaps the potential for reducing this process is reduced because of energy requirements. Alternatively, as all NEAA where shown to be taken up in amounts less than their output in milk, the use of EAA taken up in excess for *de novo* synthesis is more important, especially when feed is reduced and it may be this that controls the rate of oxidation.

In this study, the ewes were at peak lactation whereas the goats in the study by Bequette *et al.* (2001) were mid lactation (av 142 ± 20 d *post partum*). Leucine oxidation has been seen to increase with lactation (Oddy *et al.* 1988) or protein supplementation (Bequette *et al.* 1996a). Perhaps there was the potential to decrease oxidation in the goats whereas the ewes were in peak lactation and may not have the ability to decrease oxidation because leucine catabolism was needed to fulfil other functions such as supplying energy. As the ewes were also in negative energy balance, this role may have added importance.

Although the HEC decreased total protein synthesis in the mammary gland, the ratio of leucine secreted in milk protein:gland protein synthesis was similar between the insulin treated (0.65) and control ewes (0.71), suggesting that insulin did not alter the transfer of leucine into milk protein. In addition, that mammary gland protein synthesis was greater than milk leucine output (35% in the insulin treated ewes and 29% in the control ewes) suggests substantial synthesis of non-milk proteins and/or turnover of synthesised milk proteins. This is in agreement with Bequette *et al.* (1996a), who reported values of 20-59% in lactating dairy cows.

5.6 Conclusion

This experiment provides no evidence that the administration of insulin alone (by the HEC technique) increases milk protein production. However, the results demonstrated that the mammary gland was able to maintain milk protein output despite a decrease in arterial AA supply, supporting the theory that the mammary gland has the ability to directly respond to modified precursor supply. However, how this was done is unclear as there was a reduction in uptake of some EAA and no increase in mammary blood flow to compensate for these reductions in the HEC treated ewes. In addition, there was no conservation of AA within the mammary gland by reducing AA oxidation under HEC conditions, as demonstrated by the leucine kinetic calculations.

5.7 References

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Chapter 6 General discussion.

The primary aim of this thesis was to determine the role of insulin in milk protein production in lactating, pasture fed ruminants. In these studies (Chapters 3, 4 and 5), in contrast to HEC studies in concentrate-fed cows (McGuire *et al.* 1995; Griinari *et al.* 1997a; Mackle *et al.* 1999, 2000) and goats (Bequette *et al.* 2001), there was no increase in milk protein production. In the following sections the overall response to the HEC in these pasture-fed animals will be briefly summarised and possible reasons for the difference with those studies where there was a milk protein response are discussed. The final section will outline directions for future work.

Although use of the HEC technique did not increase milk protein concentration or yield in pasture-fed ruminants, it caused similar changes in AA, hormone and metabolite concentrations and supply to the mammary gland to those induced in concentrate-fed animals. Chapters 3 and 4 demonstrated that the HEC caused a decrease in circulating AA concentrations and despite a decrease in intake, utilisation of dietary crude protein for milk protein production increased in lactating ewes and cows.

In the final experiment (Chapter 5) the HEC was used in conjunction with the A-V difference technique and a labelled ^{13}-C-leucine infusion to see if insulin increased AA uptake by the mammary gland or changed the utilisation of AA for milk protein synthesis by reducing oxidation. The HEC caused a reduction in AA supply to the mammary gland through reduction in plasma AA concentrations. Furthermore, there was no evidence that blood flow was increased to compensate for this reduction. In response to the decreased AA supply, there was an increase in mammary extraction efficiency of some EAA, particularly the BCAA (Table 5-6). However, mammary uptake (mmol/h) of some EAA (isoleucine, leucine, methionine and phenylalanine) was lower in the HEC ewes.

The leucine tracer infusion (Table 5-12) indicates that while there was no reduction in leucine oxidation to conserve leucine for milk protein output.

While the study only shows the fate of leucine and no other AA in the mammary gland, this may be a mechanism whereby AA supply is maintained for milk protein synthesis when feed is restricted or in early lactation when intake is not sufficient to meet production requirements. However, such a mechanism is clearly unsustainable for any length of time and a more likely explanation, as discussed in chapter 5, is that mammary uptake of leucine was underestimated as a result of mammary blood flow being underestimated.

The three experiments presented in this thesis were not designed to discriminate between direct and indirect roles for insulin in milk protein synthesis. There are a number of alternative modes of action through which insulin could regulate mammary function. One way would be through a direct effect on the mammary epithelial cell. An indirect role could be through an indirect effect on mammary secretory cells through a secondary endocrine/paracrine mediator such as IGF-1. A third role would be through altered partitioning of substrates into non-mammary tissues restricting substrate supply for milk and milk component synthesis.

The data obtained in the present experiments support the notion that many of the effects of insulin on milk yield and composition are mediated through effects of insulin on blood substrate concentrations through effects of insulin in non-mammary tissues. Insulin caused substantial changes in plasma AA concentrations which were sufficient to make AA supply marginal for milk protein synthesis in the ewe experiments and in the Jersey cows, actually restrict milk protein output. Similarly, the effects of insulin on milk fat most likely result from a reduction in the supply of long chain fatty acids through the inhibition of adipose tissue mobilisation.

What was demonstrated in the experiments for this thesis was the ability of the mammary gland to adapt to a changing environment to maintain milk protein output. However, the question arises as to why concentrate-fed cows and goats were able to increase milk protein output under HEC conditions, compared to pasture-fed ewes and dairy cows. In the current experiments, not only were the animals fed pasture diets but difficulties were encountered in maintaining energy intakes in all experiments. This may have been the single most important factor influencing the experimental outcome.

Possible explanations for the different outcomes in the pasture- and concentrate-fed animals are discussed below.

6.1 Energy balance and effect on feed intake

One of the major differences between the studies with concentrate-fed animals versus pasture-fed animals is energy balance. Pasture-fed ewes and cows in the experiments reported in this thesis did not eat enough to match their level of production and were in negative energy balance and this is in contrast to concentrate-fed animals that were fed to requirements and reported to be in positive energy balance throughout the experiments. The energy balance data presented by Griinari *et al.* (1997b) shows that energy balance doubled by the end of the HEC.

Ulyatt (1997) suggested that pasture-fed ruminants need sufficient energy at the tissue level to increase production. So, in concentrate-fed animals, although the mammary gland was receiving signals (perhaps the decreased arterial AA concentration) to conserve AA for milk protein synthesis (by reducing AA oxidation; Bequette *et al.* 2001), in reality there was plenty of energy available and increased milk protein production resulted.

This agrees with Cant *et al.* (1999) who argues that there are set points for milk and milk component production. This is because of the tight regulation of mammary glucose uptake through transport efficiency and local blood flow, which indicates a systemically determined set point for milk production. The set point dictates fat, protein and lactose synthetic capabilities but relative rates of secretion of components can be modified by the relative supply of precursors in blood.

6.2 Genetic component

In addition to the differences in energy balance between the pasture-fed and concentrate-fed animals used in the HEC experiments, there may also be a genetic component that caused the difference in milk protein response. Animals that have been selected for milk production grazing pasture may respond differently to nutritional challenges and subsequent hormonal changes to concentrate fed animals. Several recent studies provide evidence that there are genotype x nutrition interactions between

different genotypes fed different diets. Davis *et al.* (2001) modelled the effects of nutrition (pasture vs total mixed ration (TMR)) and genotype (New Zealand vs overseas Holstein-Friesian) on lactation. The model showed that the loss of secretory capacity through quiescence of alveoli and the senescence of quiescent alveoli was greater for pasture-fed cows compared with TMR fed cows. In addition, the overseas genotype had a greater capacity to reactivate alveoli after a nutritional change. There is also evidence that there may be differences in responses to a lipolytic challenge between the New Zealand and overseas cows fed pasture or TMR, which may affect the ability to use body condition to buffer milk production in times of stress (Kolver *et al.* 2001).

6.3 Ewe as a model / species difference

That lactating ewes were used in 2 of the 3 studies in this thesis raises the question that species difference may have influenced the lack of a milk protein response, particularly as the ewes used were not a dairy breed that had been selected for milk production. However, the results from the first two experiments (Chapters 3 and 4) showed that the HEC induced the same changes with regards to circulating concentrations of AA and metabolites, and similar changes in the efficiency of dietary crude protein for milk protein output in pasture-fed ewes and cows. Therefore, the lack of a milk protein response was consistent between the 2 studies and it was felt that the ewe was an adequate model for the pasture-fed ruminant to be used for experiment 3.

On a practical basis, the ewes maintained production with the nutritional challenge imposed by the HEC compared to the cows that did not. This may or may not be because of pasture-fed ewes in New Zealand being in negative energy balance for at least the first 5 weeks of lactation (Geenty & Sykes 1986). The ewes also adapted to being housed indoors much better than the cows.

In experiment 3, the pasture-fed ewes showed similar changes with respect to AA uptake as in the concentrate-fed animals in the studies by Mackle *et al.* (2000) and Bequette *et al.* (2001). It would appear that the difference in milk protein response is because of other factors previously discussed rather than species. For this type of metabolic study, the lactating ewe is a sufficient model – the greater problem appears

more to be acclimatising pasture-fed animals to being housed indoors and, if this is important to the study, how to maintain animals in positive energy balance.

6.4 Future work

The HEC studies have clearly demonstrated that the mammary gland can adapt to a reduction in the supply of milk protein precursors by increasing extraction, mammary blood flow or reducing AA oxidation within the gland. However, use of this technique in determining a role for insulin does not allow distinction between direct and indirect effects of insulin because of the many effects insulin has on whole body metabolism. In addition, the effect on intake and satiety is confounded.

It is unclear what the signals are that control precursor supply to the mammary gland. In the HEC studies, one potential signal is the reduction in precursors such as AA but whether it is signalled from the gut (or elsewhere) or received directly as a signal by the secretory cells of the mammary gland is unknown. Given the milk protein response in concentrate fed animals was also associated with an increase in milk yield, the signal may depend on changes in both energy and AA supply. Therefore future work is needed to try to identify the signals and their source.

From the HEC experiments, there are no data to determine the effect of two periods of insulin infusion had on insulin receptors or tissue sensitivity. Or how whole-body metabolism is changed by a constant infusion of insulin creating above normal concentrations when insulin secretion is usually controlled relative to feeding. Closer examination of these areas may be important for work in the future if insulin is to be administered repeatedly in the type of experimental design used here.

It is also important to determine at the cellular level how mammary cells detect changes in concentrations of precursors and how this affects secretion. In the future this may then enable the ratio of protein to fat secreted by the cell be changed to better meet market demands.

Finally, work needs to be done to determine what controls AA transport and if these are different for individual AA, how this can be optimised. When potential signals and

pathways have been identified in cell work, it will then be time to put this back into the whole animal to see the effects on milk protein synthesis and subsequent production.

6.5 References

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Chapter 7 Appendices

7.1 Appendix A

Lactose method

7.1.1 Sample Preparation

100-200 mg of freeze-dried milk was diluted in 5 mls dH_20, mixed and put in a 37°C water bath for 15 minutes, mixing every 5 minutes. The solution was then centrifuged for 10 minutes at 2000 rpm. The supernatant was poured off into a pre-weighed tube. 2 mls of dH_20 was added to the milk tube and the mixing and centrifuge steps were repeated twice. The supernatant was analysed for lactose concentration by HPLC. Two lactose standards (5 mg/ml and 1 mg/ml) were also analysed.

7.1.2 Sample Chromatography

Lactose concentration was determined on a Shimadzu LC4A system, with an anion-cation exchange resin column (Aminex HPX-87P). A sample volume of 50 µl was injected onto the column and an elution system of a water mobile phase at a flow rate of 0.6 ml/min and a temperature of 60°C was used. Lactose was detected by a Waters refractive index detector.

7.1.3 Lactose Concentration Calculation

Concentration (mg/ml) = $Ax/As \times Cs \times DF/wt \times MW \times 10^{-3,}$

where: Ax = area of sugar in the sample,

As = area of sugar in the standard,

Cs = concentration of sugar in the standard (mM),

DF = dilution factor (volume in mls),

Wt = sample weight,

MW = molecular weight.

7.2 Appendix B

Picotag method (pers. comm.. Sarah Johnston)

Derivatisation

A stock standard solution containing 0.5 mM amino acids was prepared using 200 µl Pierce A/N (part # 20086) and 200µl Pierce B (part # 20087) standard solutions together with 0.5 mM Norleucine in a final volume of 1 ml 0.1 M HCl. Stock standard was diluted DF = 10 using 0.1 M HCl for use. For CP samples a matrix matched standard was prepared using 0.5 ml stock standard solution, 2.5 ml matrix match solution (containing 13 % w/w TCA, 0.33 % SDS, 4 mM EDTA and 7 mM DTT) in a total 5 ml.

Analysis of amino acids in samples and standards involved pre column derivatisation and chromatography modified from that described by [Bidlingmeyer, 1984 #180]. A 50 µl aliquot containing free amino acids was dried under vacuum before addition of 20 µl redry solution (2:2:1, methanol: 1M Na Acetate: TEA (under $N_{2(g)}$), then mixed by vortex and again dried down. Derivatisation solution contained methanol, MQ H_2O, TEA (under $N_{2(g)}$), PITC (under $N_{2(g)}$) (7:1:1:1) and was made fresh for each set of samples (derivatisation reagent stable for 2 hours at room temperature). Derivatisation reagent (20 µl) was added to each dried sample which were then vortex mixed and incubated at room temperature for 10 min after addition of reagent to final sample, and dried down under vacuum. Dried derivatised samples were resuspended in 200 µl diluent containing 5 % CH_3CN and 95 % phosphate buffer (5 mM Na_2HPO_4 adjusted to pH 7.40 using 10 % (v/v) H_3PO_4). Samples were vortex mixed twice and transferred to 1.5 ml microfuge tubes and centrifuged at ~14000 g for 5 min. The supernatant was transferred to autosampler vials.

Chromatography

The injection volume was set to 50 µl onto a column (in an oven set to 46°C), which was application-specified Pico-Tag C_{18} reverse phase quality controlled for rapid, high efficiency, bonded-phase separations (Waters Picotag column 3.9 X 300 mm part # WAT 010950). A UV detector set at 254 nm was used. The elution system consisted of 2 mobile phases, buffer A (70 mM Na acetate.3 H_2O adjusted to pH 6.50 using 10 % glacial acetic acid, containing 1.8 % CH_3CN, and 2.5 µl EDTA) and buffer B (15 %

aqueous methanol, containing 45 % CH_3CN). Buffer B was run in a gradient increasing from 0 % at 13.5 min to 0.5 % at 13.51 min, then by curve number 3 to 2 % at 24 min, then without curve to 6 % at 30 min, followed by an increase (curve –2) to 28.5 % at 50 min. From 50 min to 62 min buffer B increased to 36 % (curve 0) then remained at 36 % until 70.5 min when a dump step of 100 % buffer B was run to 75 min to remove residual sample from the column and the run stop was at 90 min. The flow rate was set to 1.0 ml/min. Eluents were kept under a blanket of $He_{(g)}$ to remove N_2 and O_2 from solution.

7.3 Appendix C

Uncorrected feed intake data for Table 5.1, Chapter 5.

LSmeans and standard errors (SEM) for feed intake, diet crude protein (CPI) and energy intake (MEI) on pre-infusion day and day 4 of the HEC.

	Pre-infusion		Day 4 HEC		SEM
Variable	HEC	Control	HEC	Control	
DMI	1.44 [ab]	1.47[ab]	1.31[b]	1.51[a]	0.08
CPI	302	314	279	315	15
MJ ME /day	16.4[ab]	16.9[ab]	14.6[b]	16.6[a]	0.9
Adj ME	16.4	16.9	16.7	16.6	0.9
Energy Balance	-8.1	-9.6	-7.4	-9.2	1.0
Eff diet CP utilisation	0.25	0.26	0.27	0.28	0.02

Treatments: HEC = hyperinsulinaemic euglycaemic clamp, control = BSA infused group.
LSMeans are significantly different at P<0.05, († = P<0.1).
ns = nonsignificant at P>0.1.

7.4 Appendix D

Uncorrected milk yield data for Table 5.2, Chapter 5.

LSmeans and standard errors (SEM) for milk yield, yield and concentration of milk components on the day prior to commencing the hyperinsulinaemic euglycaemic clamp (pre-infusion) and on day 4 of the hyperinsulinaemic euglycaemic clamp.

	Pre-infusion		*Day 4 HEC*	
Variable	HEC	Control	HEC[1]	Control[1]
Milk (g/d)	1429 (78)^{a}	1719 (78)^{b}	1367 (78)^{a}	1631 (78)^{b}
Solids (g/d)	236.3 (19.2)^{a}	317 (19.2)^{a}	214.1 (19.2)^{b}	284.0 (19.2)^{a}
Solids %	26.0 (0.7)^{ac}	27.7 (0.7)^{b}	23.4 (0.7)^{c}	26.2 (0.7)^{ab}
CP (g/d)	73.2 (7.8)^{a}	98.5 (7.8)^{b}	69.5 (7.8)^{a}	90.3 (7.8)^{b}
CP %	5.5 (0.1)^{a}	5.6 (0.1)^{a}	5.2 (0.1)^{b}	5.4 (0.1)^{ab}
Fat (g/d)	92.8 (6.8)^{a}	113.6 (6.1)^{b}	65.0 (6.2)^{c}	95.1 (6.1)^{a}
Fat %	6.1 (0.3)^{ab}	6.5 (0.3)^{a}	4.8 (0.3)^{c}	5.8 (0.3)^{b}
Lactose (g/d)	72.6 (4.5)^{a}	88.1 (4.1)^{b}	61.3 (4.1)^{a}	81.0 (4.1)^{ab}
Lactose %	4.8 (0.1)^{ab}	5.1 (0.1)^{a}	4.5 (0.1)^{b}	4.9 (0.1)^{a}
Individual milk proteins				
β-CN (g/l)	19.7 (0.5)	19.2 (0.5)	19.4 (0.5)	19.1 (0.5)
β-LG (g/l)	4.8 (0.3)^{ab}	5.4 (0.3)^{a}	4.4 (0.3)^{b}	4.7 (0.3)^{b}
IgG (g/l)	0.74 (0.07)^{ab}	0.82 (0.07)^{a}	0.61 (0.07)^{ab}	0.58 (0.07)^{b}
k-CN (g/l)	3.0 (0.1)^{a}	2.6 (0.1)^{ab}	2.9 (0.1)^{a}	2.4 (0.1)^{b}

[1]Treatments: HEC = hyperinsulinaemic euglycaemic clamp, control = BSA infused group.
LSMeans are significantly different at $P<0.05$, († = $P<0.1$).
ns = nonsignificant