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The Effect of a Limited Supply of Phenylalanine, Threonine, or Tryptophan on Mammary Metabolism in Dairy Cows

by

Iroshan Indikatiya Hewage

A THESIS

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Abstract

Dairy cows require amino acids (AA) in the correct amount and profile to maximize milk protein secretion. This study was conducted to examine the effects of a deficiency of 3 essential AA - phenylalanine (Phe), threonine (Thr) and tryptophan (Trp) - on mammary metabolism and milk production. Five Holstein cows were abomasally infused with water or AA solutions that contained all AA, or were deficient in Phe, Thr, or Trp. Milk and milk protein yields decreased with a deficiency of Phe or Thr but not Trp. Arterial concentrations of Phe, Thr, and Trp decreased with their respective deletions, whereas concentrations of metabolites that serve as energy substrates were unaffected by treatment. Mammary plasma flow and mammary uptake of several AA changed in response to treatments, demonstrating that the mammary gland can respond to changes in nutrient supply in an attempt to maximize milk synthesis.

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Dedication

I dedicate my MSc. thesis to my beloved parents, my wife, her parents and my two children, Adhi and Aayu. Their advice, encouragement and financial assistance laid the foundation to face all challenges confidently and achieve my goals.

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List of Abbreviations

AA	Amino acid
ADF	Acid detergent fibre
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine triphosphate
AV	Arterio-venous
BCAA	Branched-chain amino acids
BHBA	β-hydroxybutyrate
BUN	Blood urea nitrogen
BW	Body weight
C	Carbon
CP	Crude protein
CTL	Negative control
Cys	Cysteine
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EAA	Essential amino acid
GC-MS	Gas chromatography-mass spectrometry
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
His	Histidine
IE	Isotopic enrichment
Ile	Isoleucine
Leu	Leucine
LSM	Least squares mean
Lys	Lysine
MBF	Mammary blood flow
Met	Methionine
MG	Mammary gland
mM	Millimolar (concentration)
mmol	Millimole (mass)
MNE	Milk nitrogen efficiency
MP	Metabolizable protein
MPE	Mole percent excess
MPF	Mammary plasma flow
MUN	Milk urea nitrogen
N	Nitrogen
NDF	Neutral detergent fibre

NEAA	Non-essential amino acid
NE_L	Net energy lactation
No-Phe	Total amino acids without Phe
No-Thr	Total amino acids without Thr
No-Trp	Total amino acids without Trp
PDI	Protein digested in the intestine
PDV	Portal-drained viscera
Phe	Phenylalanine
Pro	Proline
Ra	Rate of appearance
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
SEM	Standard error of the mean
Ser	Serine
TAA	Total amino acids
TAA-N	Total amino acid nitrogen
TEAA-N	Total essential amino acid nitrogen
Thr	Threonine
TNEAA-N	Total non-essential amino acid nitrogen
TMR	Total mixed ration
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
WBGRa	Whole body glucose rate of appearance

Chapter 1: Review of the Literature

1.1 Introduction

Protein is the one of the most valuable components of milk. Unfortunately, the dairy cow, like other ruminants, is relatively inefficient at converting dietary crude protein (CP) into milk protein. Of the nitrogen (N) that the cow consumes, 70 to 75% is excreted in urine and feces (Bequette et al., 1998; Broderick et al., 2008), leading to potential environmental contamination. Increasing the efficiency of N conversion from feed into milk will decrease N excretion and pollution. The efficiency of incorporating dietary N into milk protein can be increased by more accurately matching the cow's supply with requirements for amino acids (AA). Even though the dairy cow is eating protein, it is the individual AA, originating from protein digestion, that are used by the mammary gland (MG) for protein synthesis; therefore, it is important that we increase our knowledge of mammary metabolism of individual AA. This chapter will focus on AA metabolism in the dairy cow, with an emphasis on Phe, Thr, and Trp where possible.

1.2 Protein Supply for the Dairy Cow

One of the goals of protein nutrition in the dairy cow is to optimize milk and milk protein synthesis with a minimum supply of dietary CP. To achieve this, the cow must be supplied with adequate rumen degradable protein (RDP) to ensure efficient rumen microbial protein synthesis, plus rumen undegradable protein (RUP) to provide AA not supplied by microbial protein.

The CP content of a feed is simple a measurement of total N content ($N \times 6.25$); it does not provide any information about RUP, RDP, or AA supply. As the name indicates, RDP is degraded in the rumen to yield AA, peptides, and ammonia, a portion of each being used for rumen microbial

growth. Rumen undegradable protein is the fraction of dietary CP that is not degraded in the rumen and is digested in the small intestine.

The cellular protein of rumen bacteria and protozoa is collectively referred to as rumen microbial protein. The synthesis of microbial protein depends on the amount of RDP, including the supply of AA and peptides, and availability of fermentable carbohydrates. Fermentation of carbohydrates provides the energy for microbial protein synthesis (Reynolds and Kristensen, 2007). Microbial protein is highly digestible and constitutes 45% to 60% of the digestive flow of protein (Clark et al., 1992). Albeit important, rumen microbial protein synthesis is not sufficient to cover the AA requirement of high yielding dairy cows and therefore RUP must also be supplied.

Although RDP and RUP are better indicators of protein supply than CP, they do not indicate the quantities of AA that are absorbed from the small intestine and are available to the cow. A better predictor of protein supply is metabolizable protein (MP). Metabolizable protein is comprised of digestible microbial protein derived from the rumen, RUP, and endogenous proteins, including sloughed cells from the gastrointestinal tract and protein secretions into the lumen of the gut [National Research Council (NRC), 2001)]. It is now considered as the standard estimation of protein supply for the dairy cow, as it gives a better indication of true protein supply (Lapierre et al., 2006). Doepel et al. (2004) reported that there is a stronger relationship between milk protein yield and MP than between milk protein yield and CP.

Due to the variety of feed ingredients that are used in dairy feed formulation and remodelling of dietary protein in the rumen by the rumen microbes, it is a challenge to determine accurately the flow of AA to the duodenum. In addition, endogenous proteins such as salivary proteins, sloughed epithelial cells, mucoproteins and duodenal enzymes also change the AA profile. Up to

20% of the duodenal flow of protein comes from the endogenous protein fraction (Ouellet et al., 2002, 2007). It is clear that the AA profile at the small intestine, the site of absorption, differs from the AA profile of the diet. In fact, dietary protein supply and requirements should be assessed for individual AA, because it is the individual AA that are used by tissues for protein synthesis, including the mammary epithelial cells for milk protein synthesis (Lapierre et al., 2006). Additionally, the metabolic fate (exclusive of incorporation into milk protein) differs among AA.

1.3 Amino Acids

Amino acids are building blocks of protein: they are critical components in milk synthesis and also have important roles in maintenance, reproduction and growth of lactating dairy cows. Twenty AA are required for protein synthesis and are divided into two groups: essential AA (EAA) and non-essential AA (NEAA). The EAA are the AA that cannot be synthesized within the animal body. Arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), and valine (Val) have been recognized as EAA for dairy cows (Schwab et al., 1975). The NEAA can be synthesized by the body; thus, there is no need to specifically provide them in the diet. For dairy cows alanine (Ala), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glutamine (Gln), glycine (Gly), proline (Pro), serine (Ser) and tyrosine (Tyr) have been identified as NEAA (Schwab et al., 1975). Specific combinations of AA are required for synthesis of each protein. If an EAA is in short supply and limits the amount of protein that can be synthesized, it is called the first limiting AA. Lysine and Met are generally considered the first limiting EAA with typical corn-based North American diets (NRC, 2001), whereas His is considered limiting in grass silage-based diets (Vanhatalo et al., 1999) or probably more with low CP diets (Lee et al., 2012).

Initially, EAA were classified based on their patterns of mammary utilization (Mepham, 1982). Histidine, Met, Phe (+ Tyr), and Trp are categorized as Group 1; their mammary uptake is equal to their output in milk protein. The uptake to output ratio of Thr does not follow a clear pattern, but tends to be similar to Group 1 (Lapierre et al., 2012). Group 2 AA consist of Ile, Leu, Lys and Val; these are extracted by the mammary secretory cells from the blood in excess relative to the amount secreted in milk protein. These excess EAA might be used as a potential energy precursor or as a source of carbon (C) and N for synthesis of other components, especially NEAA and fatty acids, in the MG (Mepham, 1982). Mammary uptake of NEAA is usually less than that required for milk protein synthesis (Mepham, 1982), suggesting synthesis of NEAA in the MG. Indeed, transfer of N of excess uptake of Lys to NEAA incorporated into milk (Lapierre et al., 2009) or in vitro (Bequette et al., 2006) has been reported.

Even though the major site of AA net utilization of the dairy cow is the MG, the ruminant liver also plays a major role in AA metabolism. After AA are absorbed across the intestine, they enter the portal blood circulation and flow through the liver prior to being released into peripheral circulation (Reynolds et al., 1992). Net portal absorption of AA is usually less than the amount digested, because catabolism of AA occurs across the portal drained viscera (PDV: gut, spleen, pancreas, mesenteric tissues and liver). The loss of Thr can be larger than those of other EAA, due to the relatively high contribution of Thr to endogenous proteins (Pacheco et al., 2006). Between 45 and 50% of the total flow of all AA absorption is removed by the liver (Lapierre et al., 2005), but generalization should be avoided as very different patterns are observed amongst AA.

The categorization of EAA into Group 1 and Group 2 also applies to AA hepatic metabolism. Group 1 AA are extensively removed by the liver, whereas Group 2 AA are minimally removed by the liver. Catabolism of AA by the different tissues is dependent on the availability of the relevant degradative enzymes in that tissue. For example, degradative enzymes of Group 1 AA are mainly present in the liver tissue (Lobley and Lapierre, 2003) whereas those for Group 2 AA are present in other peripheral tissues (Lapierre et al., 2012).

Because of the considerable AA catabolism in the PDV, namely for the branched-chain AA (BCAA: Ile, Leu, Val), the amount of free AA available for milk protein synthesis is reduced compared to that appearing in the small intestine (Lobley and Lapierre, 2003; El-Kadi et al., 2006). Once AA are extracted by the MG, catabolism of some EAA can also occur within the MG, as suggested by the net mammary uptake of Group 2 AA being higher than their milk output. Indeed, Raggio et al. (2006) reported that mammary Leu oxidation accounted for all the extra Leu taken up in excess of milk protein secretion.

1.4 Nitrogen Utilization Efficiency of Dairy Cows

The dairy cow does not convert all of the dietary N she consumes into milk N; the undigested N is directly excreted in feces, whereas another portion is excreted in urine after absorption and metabolism in the animal. These nitrogenous waste products cause N pollution by releasing large amounts of ammonia, nitrous oxide and nitrate into the air or by accumulating in water sources and the soil (Steinfeld et al., 2006). Fecal N loss includes not only undigested dietary N, but also endogenous and undigested microbial N. Fecal loss slightly varies with the supply of dietary N, but urinary N loss varies to a much greater extent with dietary intake of N (Kebreab et al., 2009). Urea excreted in urine is produced in the liver to remove excess AA and ammonia absorbed from

rumen fermentation. Therefore, urea production in the liver depends on the N supply to the rumen and post-absorptive tissue metabolism and the extent to which N supply matches tissue requirements. In previous feeding trials (Castillo et al., 2001; Frank et al., 2002), ammonia emissions were reduced by decreasing the supply of dietary CP without negative effects on milk yield.

The conversion of feed N into milk protein is referred to as milk N efficiency (MNE). This is a gross measurement, being the ratio of the amount of N secreted into milk divided by the amount of N consumed from the diet. Overall, MNE is around 0.25 to 0.30, with the remainder of the N intake excreted in urine and feces (Spears et al., 2003; Nadeau et al., 2007). Efficiency of utilization of dietary N in dairy cows can be increased by 1.5 to 2 percentage units with every 1 percentage unit decrease in dietary CP concentration (as cited in Nadeau et al., 2007); therefore, reducing dietary protein supply is a possible way to reduce N excretion but will only be accepted by dairy producers as long as milk protein synthesis is not negatively impacted. Feeding dietary protein below the cow's requirement will result in a negative milk protein response. By balancing AA supply with requirements, MNE can be increased, but there is an upper biological limit. Dijkstra et al. (2013) indicated that the maximal efficiency that can be achieved is 0.43; this calculation was based on a 650 kg cow producing 40 kg/d of milk and consuming 24.1 kg of dry matter (DM). Those authors explained that this maximum efficiency is the result of inevitable N losses such as undigestible N, catabolism of absorbed AA and endogenous N loss.

Marginal efficiency is the increase in milk protein that results from each additional unit of dietary protein. Accurate prediction of a milk protein response with dietary protein supplementation is one of the challenges in dairy feeding, due to changes in the AA profile of the

CP as a result of ruminal fermentation. The NRC (2001) uses a fixed efficiency rate of conversion of MP for milk production; however, individual studies show that marginal efficiency is considerably lower compared to the NRC model (Doepel and Lapierre, 2010; Haque et al., 2012; Dijkstra et al., 2013). Doepel et al. (2004) also demonstrated that the marginal efficiency of MP into milk protein is not constant and varies with each individual AA. Hanigan et al. (1998), using data from 7 casein infusion studies, reported that only 21% of the postruminal supply of casein was recovered as milk protein. This marginal efficiency is low compared to the NRC (2001) fixed efficiency of 67%.

Although milk protein output increases with postruminal AA supplementation, the marginal efficiency of N utilization decreases. For example, Guinard and Rulquin (1994) reported that the efficiency of conversion of absorbed protein into milk protein decreased when the digestible protein supply was increased from 1403 g/d to 2073 g/d. Whitelaw et al. (1986) reported that infusing casein (200 g/d) resulted in an 81 g/d milk protein increment (efficiency of 40.5%) whereas casein infusion at 600 g/d only gave a 158 g/d milk protein response (efficiency of 19.3%). Studies that decreased the supply of protein have shown increasing efficiency of N utilization (Castillo et al., 2001). The increased efficiency with the lower supply of AA could be due to a reduction of AA catabolism in the body (Lapierre et al., 2006).

1.5 Amino Acid Uptake and Mammary Metabolism

The supply and uptake of nutrients by the MG are crucial for milk synthesis. Amino acids, glucose, acetate, β-hydroxybutyrate (BHBA), lactate and long chain fatty acids that are available in the blood are used as building blocks and energetic precursors for synthesis of milk components. There are 3 sources of AA for the MG: plasma proteins, peptides and free AA; however, on a net

basis, it is primarily free AA in the plasma that are used to synthesize milk proteins (Mepham and Linzell, 1966). Not only are these free AA used to synthesize milk protein, they are also used for enzyme and structural protein synthesis. However, if there is no growth of the MG, protein degradation is in equilibrium with protein synthesis, with no net demand of free AA to support the latter process. In addition, EAA will also be involved in energy production and NEAA synthesis in the MG.

When considering catabolism of AA in the MG, transamination and deamination are the main processes. During transamination, the amine group of one AA transfers to a keto acid to form a new AA and a different keto acid. Deamination is removal of the amine group from an AA. For example Glu, which is produced by transamination of Ala, can be deaminated to form α-ketoglutarate, an intermediate in the citric acid cycle. Regarding NEAA synthesis, Asp, Pro, Glu, and Arg are synthesized using citric acid cycle intermediates and N atoms from NH^{4+}. Other NEAA (Cys, Gly, Ser, Ala) are synthesized by using glycolysis intermediates such as pyruvate and 3-phosphoglycerate. Previous infusion studies of Lys and Leu demonstrated that their excess uptake by the MG is oxidized and NH^{4+} (one of the end products) contributes to synthesis of NEAA (Bequette et al., 1996; Mabjeesh et al., 2000). However, other studies have shown that mammary uptake of Phe, Met, Thr and His equals their milk output, confirming that they are mainly used in milk synthesis (Guinard and Rulquin, 1994; Metcalf et al., 1996). Guinard and Rulquin (1994) conducted a Lys infusion study to examine the mammary uptake pattern of AA and found that EAA arterial concentrations linearly increased with increasing amounts of Lys and that mammary uptake of Lys was linearly increased. Bequette et al. (2000) observed a 90% reduction of His

arterial concentration, and a slight reduction of net His mammary uptake when lactating goats were infused with a mixture of AA deficient in His relative to a mixture containing all AA.

Mammary metabolism of AA can be examined using the following measures: mammary arterio-venous (AV) concentration difference, mammary fractional removal, mammary net uptake and the ratio of mammary uptake to milk output. Fractional removal represents the proportion of the nutrients taken up by the MG relative to the total amount seen, or presented, to the MG. This is calculated as the arterio-venous difference divided by the arterial concentration of the AA. Net AA uptake by the MG can be calculated as mammary plasma flow (MPF) multiplied by the AV difference; i.e. uptake of AA (mmol/h) = AV difference of AA (mM) × MPF (L/h). The net intra-mammary AA utilization can be evaluated quantitatively through examination of the ratio of mammary uptake to milk output ratio. As previously mentioned, mammary uptake of Group 1 AA, i.e. His, Met, Phe, Tyr and Trp, is usually quantitatively transferred into milk protein. However, mammary uptake of Group 2 AA is in excess compared to their output in milk protein.

1.6 Mammary Plasma Flow

There is a strong correlation between MPF and milk yield of the dairy cow (Linzell, 1974). The supply of substrates for milk synthesis is regulated to a certain extent by the MG through MPF; however, it is not clear if MPF has direct control of milk synthesis or whether a change in MPF is an indirect response to changes in mammary metabolism. Recent studies suggest changes of MPF occur according to the metabolic need in the MG (Lacasse and Prosser, 2003).

Regardless of AA plasma concentration, the MG is capable of adjusting AA uptake by changing its blood flow; therefore, the profile of AA in the blood circulation has an influence on the blood flow to the MG. Bequette et al. (2000) reported that the lactating goat MG is capable of

responding to a deletion of His from an abomasal infusion of a complete AA mixture by increasing the rate of mammary blood flow (MBF). Despite a severe decrease in arterial plasma His concentration with the His deletion (8 vs. 73 μM), milk output of His decreased only by 18% as a result of the 33% increase in MBF and a large increase in mammary fractional removal of His. When the His deficiency was reversed, MBF decreased. This inverse relationship between MBF and His supply indicates how the MG attempts to compensate for low AA arterial concentrations. Raggio et al. (2006) reported a reduction of plasma flow with a duodenal casein infusion which could be due to the MG adapting to the excess AA supply. Guinard and Rulquin (1995) reported a similar response: they infused graded amounts of Met into the duodenum and observed reductions of MPF with the increased Met supply.

A reliable method for measuring MPF is necessary for determining mammary uptake of nutrients. Different methods are available. An ultrasonic flow probe can be placed around the external pudic artery (e.g. Thivierge et al., 2002) or downstream dilution of para-amino hippurate can be used (Metcalf et al., 1992). Estimation of MPF using the Fick principle (Fleet and Mepham, 1983) is the most common method, primarily because of the ease of using this method. The Fick principle states that MPF of an organ can be calculated using a marker substance assuming that no catabolism or use of the marker substance occurs across the tissue. In dairy cows, Phe + Tyr are used as marker substances. On a net basis, Phe is not metabolized within the MG for purposes other than milk protein secretion, but Phe can be converted into Tyr within the MG (Lemosquet et al., 2010a); therefore, the total of Phe + Tyr is used in the Fick principle process (Verbeke et al., 1972). Mammary uptake of Phe + Tyr is equal to output in milk. Therefore, by measuring Phe and Tyr milk output and their AV difference, MPF can be calculated by using the equation: MPF =

Net output/AV difference. Once MPF is determined, then net mammary uptake for a nutrient is calculated as MPF × AV difference of that nutrient.

Representative blood samples should be collected to obtain accurate nutrient uptake values. The main arterial blood supply to the MG is from the external pudic artery, whereas venous blood drains via the subcutaneous abdominal vein (milk vein), external pudic vein, and perineal vein (Fleet and Mepham, 1983). Arterial blood can be collected from a coccygeal vessel as it is very convenient and blood from either a coccygeal vein or artery is considered to representative of arterial supply, as metabolism across the tail is negligible (as cited in Cant et al., 1993). The milk vein can be used as a sampling site for venous blood, as it is assumed that these samples are representative of blood exclusively from the MG. Increasing the frequency of feeding and having the cow in the same posture (standing position is recommended) during blood sampling is recommended when MPF is estimated using the Fick principle (Bequette et al., 1997).

1.7 Limiting Amino Acids and Infusion Studies

Milk protein synthesis of the dairy cow can be limited by the particular AA that is in shortest supply relative to the cow's AA requirement. The efficiency of utilization of absorbed AA is determined by the supply of the first limiting AA. The benefits of balancing diets to supply an ideal profile of AA are enhanced milk and milk component yields, and improved use of dietary protein.

The AA profile in the digested protein will differ from that of the diet through action of the rumen microbes. Therefore, analyzing the profile of the diet does not give an adequate picture of the true supply of AA and does not indicate which AA would be limiting for milk protein synthesis. In addition, anabolic and catabolic reactions of the individual AA at different tissues also affect

determination of the limiting AA for milk synthesis. A particular AA only accumulates in the plasma after its requirement is met; therefore determination of AA plasma concentration can be a valuable tool for predicting the limiting AA for milk production under specific conditions.

Schwab et al. (1992) identified Met and Lys as the most limiting EAA for milk synthesis in dairy cows fed typical North American diets. This has been confirmed by other studies (Rulquin et al., 1993; Robinson, 1995). Varvikko et al. (1999) examined lactation and metabolic responses of the dairy cow to abomasal infusions of increasing amounts of Met and Lys. In that study, cows were fed grass silage and a cereal supplement. The treatments were 0, 10, 20, 30, or 40 g/d of Met (Experiment 1) and 0, 15, 30, 45, or 60 g/d of Lys (Experiment 2). Treatments had no effect on either milk protein yield or milk protein concentration. The results of this study indicated that Met and Lys were not the first limiting AA when cows were fed a diet containing grass silage and cereal supplement.

In general, the additional supply of Met or Lys through infusions mainly affects the casein fraction of milk protein and the milk protein response is more prominent than the milk yield response (Rulquin et al., 1993). Although the response to Met, Lys or His infusion supply into the duodenum on dairy cow performance might have varied across studies, a common response, even with different basal diets, was reported: plasma concentration of infused EAA was increased, and mammary fractional removal of infused EAA was decreased (Guinard and Rulquin, 1995; Korhonen et al., 2000; Rulquin and Pisulewski, 2000).

Vanhatalo et al. (1999) identified His as a limiting AA for grass silage-based diets. They examined the milk production response to abomasal infusion of 6.5 g of His alone or in combination with either 6.0 g of Met or 19.0 g of Lys, or both. In that study, cows were fed a diet

of grass silage and cereal supplement. Milk and milk protein yields increased with the His alone treatment and did not increase further when His was combined with the other AA. This infusion study confirmed that His was the first limiting AA when cows were fed a diet with grass silage and cereal supplement. Subsequent infusion studies (Kim et al., 1999; Vanhatalo et al., 1999; Huhtanen et al., 2002) confirmed this result. Further work, however, indicated that the limitation might have been related more to the low protein supply diet rather than the grass silage base per se (Lee et al., 2012). Metabolizable protein supply from low CP rations is characterized by a high proportion of microbial proteins that have a low concentration of His (NRC, 2001).

1.8 Amino Acid Deletion Studies

Rather than infusing a specific AA on the top of a defined ration, the potential effect of one individual AA on mammary metabolism can be examined by supplying all AA and comparing with the same mixture deleting the specific AA under study. This is an effective method, because a deficit of the specific AA induces a larger response than an excess supply of that AA.

Deletion studies conducted with Group 1 EAA indicated that the deletion of His or Met (Weekes et al., 2006) and His, Met or Phe (Fraser et al., 1991; Doelman et al., 2013) negatively affected milk protein secretion. In dairy cows receiving as the sole source of protein a post-ruminal infusion of casein, extra EAA were supplied as a free AA infusion: deletion of His decreased milk yield and deletion of Phe tended to decrease milk yield, whereas deletion of Met decreased productive N (sum of milk protein yield and N retention; Fraser et al., 1991). In early lactating cows fed a 9% CP diet and receiving 1.1 kg/d of an abomasal infusion of an AA mixture without His or Met, Weekes et al. (2006) reported an increment of milk protein yield when the His or Met deletion was corrected. In the study of Doelman et al. (2013), early lactation cows were fed a diet

that supplied 65% of the cows' MP requirement and 100% of the net energy lactation (NE_L) requirement and were given an abomasal infusion of saline, all EAA, EAA less Phe, EAA less His, EAA less Met, or EAA less Trp. Milk protein yield increased by 22% in the all EAA infusion group compared to the saline treatment and milk yield was decreased by 15.7, 23.6 and 22.6% in the Met, Phe and His deletion treatments, respectively, compared to the all EAA group. For the His, Met and Phe deficiency groups, milk protein concentration was reduced by 0.25, 0.36 and 0.37 percentage points, whereas with the Trp deficiency treatment, there was no significant effects on milk yield or protein composition compared to all EAA (Doelman et al., 2013).

Results are more equivocal for Group 2 AA especially for the BCAA. In the same study reported above, Fraser et al. (1991) reported no effect on milk and productive N when deleting as a bulk group Arg, Ile, Leu, Val and Tyr. Similarly, Weekes et al. (2006) did not observe a reduction of milk protein yield or concentration with the infusion of an AA mix lacking the three BCAA relative to a positive control. However, Doelman et al. (2014) fed an 11.7% CP diet and infused a complete EAA mixture, or an EAA mixture devoid of Lys, Leu or BCAA to determine the effect of these AA on milk protein synthesis. An 18% increase in milk protein yield was observed for the EAA infusion compared with the control treatment. With deletion of Lys, Leu and BCAA from the infusion, milk yield was decreased by 10.2, 21.1 and 12.2%, respectively, relative to the EAA group. Milk protein concentration was also reduced by 0.23, 0.30 and 0.29 percentage points for Lys, Leu and BCAA, respectively, compared with the EAA group. In the study of Fraser et al. (1991), described above, deletion of Lys decreased milk and milk protein yields. Lapierre et al. (2009) reported that Lys deletion from a mixture of all AA infused into the abomasum numerically

decreased milk protein yield with no effect on milk yield in dairy cows fed 70% of their MP requirement.

Doepel and Lapierre (2011) infused a mixture of EAA, with or without Arg, post-ruminally into lactating cows fed 72% of their MP requirement and did not observe negative effects on milk and milk component yields in the Arg deletion group. These results suggested that Arg supply from the diet together with synthesis by the cow was sufficient in the Arg deletion group; however, these cows were fairly late in lactation and it is quite possible that the effects would have been different in early lactation cows. Again, in the study of Fraser et al. (1991), deletion of Cys, Tyr or Thr did not affect cows' performance.

1.9 Relationship Between AA and Energy Metabolism

Amino acid metabolism includes AA biosynthesis and catabolism. Catabolism of AA involves removal the amino group by transamination or oxidative deamination; the C skeleton is then converted into metabolites, mainly pyruvate, acetyl CoA, and acetoacetyl CoA that can act as intermediates in the Krebs cycle. These metabolites are then used for energy production, gluconeogenesis, or conversion to triglycerides. The N atoms of AA are used for biosynthetic processes of other N-containing compounds, such as NEAA and nucleic acids, or excreted as urea.

When considering energetic reactions through the Krebs cycle, there are two classes of AA: glucogenic and ketogenic. Glucogenic AA refer to AA with C skeletons that are converted to Krebs cycle intermediates that can be used to synthesize glucose. The ketogenic AA are AA with C skeletons that can only be converted to acetyl CoA, acetoacetyl-CoA or acetoacetate and cannot directly contribute to glucose synthesis. Among the EAA, His, Met, Thr, Val are categorized as

glucogenic; Leu and Lys are ketogenic EAA; and Phe, Trp and Ile have both glucogenic and ketogenic abilities.

Glucose is the major energy source of the dairy cow and is the main precursor for lactose synthesis in the MG (Bickerstaffe et al., 1974). Glucose requirements of ruminants are largely met through gluconeogenesis, which predominantly occurs in the liver using glucose precursors absorbed following fermentation and digestion of nutrients derived from the diet. The main glucogenic precursors are propionic acid, glucogenic AA, and lactate (Danfaer, 1994). Contribution of AA to glucose synthesis is estimated to range from 5 to 30% (Reynolds et al., 1992; Lozano et al., 2000).

Milk lactose yields were increased with an abomasal infusion of casein, but not with a postruminal infusion of glucose or propionic acid (Clark et al., 1977; Lemosquet et al., 2009a). Interestingly, infusion of propionate increased milk protein yield (Raggio et al., 2006). This suggests there are common metabolic pathways linking protein and energy metabolism. Indeed, many metabolites like AA, glucose, acetate, BHBA and triglycerides are taken up and metabolized within the mammary tissue and involved with milk protein, lactose and fatty acid synthesis. With increased protein or energy supply of the dairy cow, availability of nutrients for mammary metabolism is highly variable, but the MG is very flexible and capable of metabolic adjustments to sustain milk protein and lactose secretion (Lemosquet et al., 2010b). Nutrients taken up by the MG are not only used in the synthesis of milk components but can also be oxidized and produce adenosine triphosphate (ATP) to supply the energy needed to support milk synthesis. Short supply of energy may limit milk protein secretion, as production of ATP and its availability might be the limiting factor for milk protein synthesis, as 4 or 5 ATP are needed to support the synthesis of 1

peptide bond. Also, in a situation of energy and/or glucose deficiency, AA could be used for hepatic glucose synthesis, leading to a reduction in the availability of AA for milk protein synthesis.

Mechanisms underlying the response in milk protein yield may also differ depending on the type of nutrient supply. With an increased supply of casein or glucogenic substances, an increment in milk protein occurred with a parallel increment in mammary uptake of Group 1 AA (Lemosquet et al., 2010b). However, with the casein supply, this increment of mammary uptake of Group 1 AA was linked to an increment of mammary AV difference and arterial concentration of those Group 1 AA, whereas with the increased supply of glucogenic substances, this increment of mammary uptake was associated with a tendency for an increment of MPF (arterial concentration or AV difference of Group 1 AA was not changed). Similarly, the increased milk lactose yield in response to protein supply was not related to increased glucose mammary uptake, indicating that within the MG, different pathways were regulating the proportion of glucose taken up which was directed towards lactose (Lemosquet et al., 2010b).

In addition, with the increased supply of casein, milk fat yield was increased (Lemosquet et al., 2007), but with the increased supply of glucogenic substances excluding AA, milk fat yield was decreased (Rigout et al., 2002; Lemosquet et al., 2009a, 2009b). The increment of milk fat yield with the increased supply of casein mainly occurred through increasing mammary uptake of BHBA (Lemosquet et al., 2010b).

It is obvious that glucose is the molecule that makes the link between lactose synthesis and protein synthesis. When an increment of glucose availability in the body occurred due to an increased supply of protein, lactose yield was also increased, but when glucose availability

increased only with energy supply, then lactose yield was not stimulated in the dairy cow (Lapierre et al., 2010). The reason for this discrepancy is not clear, but this could be due to EAA stimulating the production of enzymes related to lactose synthesis. Vanhatalo et al. (2002) conducted an abomasal infusion study with early lactation dairy cows. The four treatments were continuous abomasal infusions of water, casein at 300 g/d, glucose at 300 g/d, and casein + glucose each at 300 g/d. Milk protein and lactose yields increased with the casein and the glucose infusions, with an additive effect with the infusion of casein + glucose. Increased protein yield with the glucose infusion could be due to sparing AA from hepatic utilization. The increase in lactose yield associated with casein and glucose infusion relative to the glucose only infusion indicates that if AA supply is inadequate, lactose synthesis is limited.

1.10 Whole Body Glucose Rate of Appearance

In the dairy cow, glucose is the main precursor for mammary lactose synthesis. The MG cannot synthesise its own glucose and therefore glucose must be extracted from the blood for lactose synthesis. Glucose supply to the MG could be a limiting factor for milk lactose synthesis, and theoretically glucose availability at the body level might have an impact on synthesis of lactose in the MG. Glucose availability at the whole body level is measured as whole body glucose rate of appearance (WBGRa). It represents all the glucose inflows (intestinal absorption, gluconeogenesis and glycogenolysis) into the blood pool. The majority of glucose available to the dairy cow originates from gluconeogenesis, with up to 25% of WBGRa derived from dietary absorption when utilization of glucose by the PDV contribution is correctly accounted for (Galindo et al., 2011).

Two metabolic functions affect the measurement of the net flux of glucose: absorption of glucose from intestinal starch digestion results in a positive flow, whereas glucose utilization by the gut from the arterial glucose supply results in a negative flow. The net flow is the sum of these two events. Therefore, a reduction of glucose usage by the PDV will change the net portal glucose flux. The digestive tract of the dairy cow also catabolizes AA. Extra supply of AA could theoretically act as an energy source for the PDV with, as a result, a drop in the utilization of glucose as an energy source. This could leave extra glucose to be used in peripheral tissues such as the MG. However, increased AA or casein supply did not alter glucose utilization by the PDV in sheep (El-Kadi et al., 2006) or in dairy cows (Galindo et al., 2011). Similarly, infusion of 300 g/d of Gln did not alter glucose net flux across the PDV, suggesting little, if any, sparing effect of glucose utilization by the PDV with increased supply of AA (Doepel et al., 2006). In dairy cows, liver glucose synthesis contributes 82% of WBGRa, but very little to the whole body utilization (less than 3%), because the liver uses fatty acids as a main energy source (Lapierre et al., 2010). In dairy cows, approximately 50 to 60% of whole body glucose utilization occurs in the MG (Lemosquet et al., 2009b). This suggests that milk yield would be directly related to whole body glucose availability and that glucose availability at whole body level could be the limiting factor for milk production. However, when glucose availability was increased through energy supply, milk lactose yield did not increase, whereas when increased glucose availability was induced by an increase in protein or AA supply, lactose yield did increase (Lapierre et al., 2010). This indicates that WBGRa was not the driving force for lactose synthesis.

The fluxes of glucose through different tissues and at the whole body level can be quantified using infusion into blood of glucose labelled with stable isotopes. The stable isotopes are atoms of

the same element that differ in atomic mass due to differences in the number of neutrons contained in the atoms. For example, [6, 6-$^{2}H_2$] glucose contains substitution by two deuterium of the hydrogen atoms on the 6th C of a glucose molecule. The hypothesis underlying the use of isotopes to study nutrient kinetics is that the labelled nutrient is functionally identical to the unlabelled nutrient. It is also assumed that blood is a single pool in the body into which is made the infusion of labelled glucose, from which the sampling is done, and into which release of unlabelled glucose occurs. Bolus injection and constant infusion of stable isotopes are the most common techniques used to assess glucose kinetics (Wolfe et al., 2005). With the constant infusion method, the nutrient labelled with stable isotopes is infused at a constant rate until an isotopic equilibrium is reached. At equilibrium (steady state), the glucose rate of appearance in the plasma is equal to the rate of disappearance. Due to the molecular mass difference between the unlabelled glucose and labelled glucose, the relative abundance of labelled glucose, the isotopic enrichment (IE), can be measured by gas chromatography-mass spectrometry (GC-MS). Glucose rate of appearance (Ra) can be derived from the plasma IE of glucose calculated by: $Ra = i \times (IE_{inf}/IE_{pl} - 1)$, where i is the infusion rate of the labeled tracer, IE_{inf} is the IE of glucose in the infusate and IE_{pl} is the IE of glucose in the plasma at plateau, respectively.

In dairy cows, depending on the type of glucogenic nutrient supply, WBGRa increased with different efficiencies. Lemosquet et al. (2009a) examined WBGRa and milk lactose responses in dairy cows receiving isoenergetic duodenal infusions of glucose, a mixture of 5 NEAA or propionic acid. The WBGRa was increased with all the energy yielding nutrients compared with the control treatment. For control, glucose, propionate, and NEAA mixture, average WBGRa was 502, 745, 600, and 576 mmol/h respectively. The WBGRa was higher with the glucose infusion

compared with infusion of propionic acid or NEAA. Lactose yield was slightly increased with glucose or propionic acid infusion relative to CTL, whereas when NEAA were infused, lactose yield was decreased despite increased WBGRa.

Previous studies reported that postruminal infusions of casein increased WBGRa in dairy cows (e.g. Clark et al., 1977; Lemosquet et al., 2009b). In theory, this could result from duodenal epithelial cells using AA as energy rather than using glucose from lumen starch digestion, or more likely from increased glucose production in the liver, kidney, or both, from AA. In cows in established lactation, the increment in WBGRa induced by AA infusion was matched with the increment in glucose net hepatic release (Galindo et al., 2011).

Rigout et al. (2002) suggested that increased WBGRa could be an important factor to increase mammary glucose uptake and subsequently to increase milk production. However, mammary glucose uptake or milk lactose yield does not represent a fixed proportion of WBGRa. Indeed, mammary glucose uptake was not changed with increased AA supply, despite increased lactose yield (Lemosquet et al., 2009b; Galindo et al., 2011). Conversely, glucose uptake tended to increase with propionate infusion, with no effect on lactose yield (Lemosquet et al., 2009a), suggesting that the MG has its own control for regulating mammary glucose uptake and glucose utilization. Therefore, glucose synthesis and mammary glucose uptake and utilization for lactose synthesis seem to depend on the relative balance between glucogenic nutrient availability and the metabolic demand of the MG (Lemosquet et al., 2009a).

1.11 Research Rationale and Objectives

It appears that a limited supply of EAA will negatively affect milk protein synthesis. Threonine, Trp and Phe are usually taken up by the MG in the same amount as that used for milk

protein secretion. As a result, we can raise the question "will milk protein synthesis decrease if the supply of these AA is limited?" and then hypothesize that a limited supply of these AA will negatively affect milk protein output and mammary metabolism.

The MG has a great ability to alter blood flow, as well as fractional removal to extract AA from blood for a more or less constant supply of AA for milk protein synthesis when blood concentrations are low (Bequette et al., 2000; Vanhatalo et al., 2002). With this premise, we can raise the question of how the MG adapts when the supply of Phe, Thr or Trp is decreased. We don't know how a limited supply of these 3 AA will affect WBGRa of glucose. Our third question is: how will a shortage in the supply of one AA affect WBGRa and how is this related to milk lactose secretion?

Hypotheses

The hypotheses of the present study are that a limited supply of Phe, Thr or Trp in lactating dairy cows will:

- Decrease mammary uptake of these AA, but increase fractional removal
- Decrease milk protein synthesis
- Increase whole body rate of appearance of glucose.

Objectives

The objectives of this project are to determine:

- how milk and milk component yields change with a limited supply of Phe, Thr or Trp
- how net mammary uptake of AA and energy substrates changes with this limited supply of EAA

- the relationship between AA deficiencies and glucose availability and mammary uptake and utilization for lactose synthesis.

Chapter Two: The effect of a limited supply of phenylalanine, threonine, or tryptophan on milk production, mammary metabolism, and whole body glucose kinetics

2.1 Introduction

The dairy cow is only 25-30% efficient in converting dietary protein to milk protein (Bequette et al., 1998; Broderick et al., 2008). This inefficiency is attributed to incomplete protein digestion in the gastrointestinal tract, absorption of dietary CP as ammonia and catabolism of absorbed AA, the latter resulting in incomplete usage of absorbed AA. Milk protein synthesis in the dairy cow is dependent on a sufficient supply of each EAA reaching the MG. Ensuring the correct supply and profile of EAA is a challenge. Extensive rumen fermentation by the microbial flora drastically changes the AA composition of the duodenal protein flow compared to the AA composition of the diet; therefore, it is a challenge to accurately predict the digestive flow of AA and estimate EAA requirements. It is generally accepted that in typical North American diets, the first and second limiting AA are Lys and Met (NRC, 2001), whereas in grass-based diets, His would be first limiting (Varvikko et al., 1999) although it has been argued that limitation for His might be more related to a low CP diet, usually involving a high proportion of microbial protein having a low concentration of His (Lee et al., 2012). Although much of the AA research in dairy cows has focused on these 3 EAA, other EAA could also limit milk protein synthesis if their provision was inadequate. Through deletion studies, the effect of the BCAA, together or individually, has also been recently studied (Rulquin and Pisulewski, 2006a; Weekes et al., 2006; Haque et al., 2013) as well as the effect of Arg (Doepel and Lapierre, 2011; Haque et al., 2013).

The current research project focuses on 3 EAA for which research, especially conducted as deletion studies, is rather limited: Phe, Thr and Trp. The recommendation by Rulquin et al. (2007) for the proportion of each AA relative to the protein digested in the intestine (PDI) is 4.6, 4.0 and

1.7%, for Phe, Thr and Trp respectively. Interestingly, estimated average proportions of the MP supply from all control treatments from Doepel et al. (2004) averaged 5.0, 4.9 and 1.2% (Lapierre et al., 2008) and as such would be higher than recommendations for Phe and Thr. Recently, Doelman et al. (2013) reported that deletion of Phe from an EAA infusion decreased milk yield by 24% compared with a total EAA infusion. Furthermore, Rulquin and Pisulewski (2006b) reported a linear increase of milk protein yield when the proportion of Thr relative to PDI increased from 3.65 to 6.0%.

Generally, in response to AA infusion, milk protein and lactose yields increase. Amino acids are one of the glucose precursors in dairy cows and indeed, WBGRa increased in dairy cows with an additional supply of AA (Lapierre et al., 2010). There is clearly a relationship among AA supply, glucose availability at the whole body level, mammary utilization of glucose and mammary lactose synthesis. Therefore, the hypotheses are that a limited supply of Phe, Thr or Trp will decrease mammary uptake of these AA, and decrease milk yield and milk protein and lactose yields despite an increased WBGRa. The objectives of the current study were to examine the effect of a limited supply of Phe, Thr or Trp on mammary metabolism of AA and energy-yielding nutrients in relation with WBGRa.

2.2 Materials and Methods

2.2.1 Cows

The study took place at the Dairy Research and Technology Centre at the University of Alberta (Edmonton, AB, Canada). Animals were cared for according to the Canadian Council on Animal Care (2009) guidelines and all experimental procedures were approved by the Animal Care and Use Committee of the University of Alberta. Five second-lactation Holstein cows were used.

Average body weight (BW) and days in milk (DIM) at the beginning of the experiment were 545.8 ± 15.5 kg and 63 ± 1.6 d, respectively. Two abomasal catheters were implanted in each cow at 37 ± 0.8 DIM, as described by Doepel et al. (2006). In brief, 1 h before the surgery, cows were given 3 mg/kg of ketoprofen (Anafen®) and 20 mg of acepromazine maleate (Atravet®) intramuscularly. The right flank of the cow was shaved between the dorsal and ventral midlines and from the point of the elbow to the hook bone and was prepared for laparotomy using Hibitane surgical scrub and 70% isopropyl alcohol. A paravertebral block was then performed using lidocaine HCL 2% with epinephrine. After entering the abdominal cavity, the pyloric portion of the abomasum was exteriorized and a 0.5 cm long incision was made through the abomasal wall. The catheter was inserted into the incision, sutured in place using surgical mesh, and exteriorized through the dorsal surface of the cow between the lumbar transverse apophysis. After that, a standard omentopexy was performed.

Cows were housed in tie stalls for the duration of the experiment, and had free access to water. They were milked twice daily at 0400 and 1500 h.

2.2.2 Treatments and Diet

The experimental design was a 5 × 5 Latin square. Each period was 10 d long, with 7 d for adaptation and 3 d for measurement. Each period was followed by a 4 d inter-experimental period in which the cows did not receive treatments. This protocol was used to ensure no treatment carry-over effects from period to period. The treatments were abomasal infusions of: 1) 15 L of water (CTL: negative control); 2) a mixture of all AA (TAA: positive control) supplied at a rate that the diet plus the infusion supplied 100% of the requirements for MP; 3) TAA without Phe (No-Phe); 4) TAA without Thr (No-Thr) and 5) TAA without Trp (No-Trp). The AA composition of the

infusion was the same as that in milk casein (Table 2-1). The AA infusates were prepared every 2 to 3 d. They were delivered in 15 L/d of water and infused continuously via peristaltic pump.

Table 2-1 Daily amount of amino acids (AA) infused abomasally into cows for each treatment (g/cow/d)

AA	Treatment[1]			
	TAA	No-Phe	No-Thr	No-Trp
Alanine	27.7	27.7	27.7	27.7
Arginine	33.3	33.3	33.3	33.3
Asparagine	34.3	34.3	34.3	34.3
Aspartate	27.8	27.8	27.8	27.8
Cysteine	3.6	3.6	3.6	3.6
Glutamate	52.9	52.9	52.9	52.9
Glutamine	135.0	135.0	135.0	135.0
Glycine	16.7	16.7	16.7	16.7
Histidine	24.8	24.8	24.8	24.8
Isoleucine	44.8	44.8	44.8	44.8
Leucine	83.8	83.8	83.8	83.8
Lysine	70.5	70.5	70.5	70.5
Methionine	26.0	26.0	26.0	26.0
Phenylalanine	46.3	----	46.3	46.3
Proline	95.5	95.5	95.5	95.5
Serine	51.4	51.4	51.4	51.4
Threonine	39.0	39.0	----	39.0
Tryptophan	10.8	10.8	10.8	----
Tyrosine	49.6	49.6	49.6	49.6
Valine	51.0	51.0	51.0	51.0
Total	924.8	878.5	885.8	914

[1]TAA = all AA infused; No-Phe = all AA excluding Phe; No-Thr = all AA excluding Thr; No-Trp = all AA excluding Trp.

Cows were fed a diet formulated to supply 100% of the NE_L requirement and 70% of the MP requirement for a second lactation cow weighing 550 kg and producing 40 kg milk/d with 3.6% fat and 3.2% protein (NRC, 2001; Table 2-2). Although formulated to supply these amounts, the diet supplied 97% of the NE_L requirement and 66% of the MP requirement when supply was estimated with the actual ingredient composition of the experimental diet. During the 4-d inter-

experimental periods, the total mixed ration (TMR) was top-dressed with soybean meal (2 kg/d, DM basis) so that the diet met 100% of the MP requirements.

Table 2-2 Ingredient and nutrient composition of experimental diet

Ingredients	% of diet DM
Alfalfa hay	10.0
Barley silage	42.0
Corn grain, rolled	24.7
Barley grain, rolled	10.1
Barley grain, ground	5.1
Wheat distillers grains	2.1
Rumen-protected fat[1]	2.0
Tallow	1.3
Molasses	1.1
Sodium bicarbonate	0.71
Dicalcium phosphate	0.36
Vitamin/mineral premix[2]	0.18
Salt	0.18
Magnesium oxide	0.18
Nutrients	
NE_L, Mcal/kg[3]	1.68
CP, %	12.0
ADF, %	19.3
NDF, %	31.6
Calcium, %	0.71
Phosphorus, %	0.39
Magnesium, %	0.26
Potassium, %	1.62
MP, g/day[3]	1715

[1]Megalac, Church & Dwight Co. Inc, Princetown, NJ, USA

[2]Supplied the following per kg of diet: 1.19 mg Co, 36.9 mg Cu, 1.44 mg I, 64.8 mg Mn, 0.30mg Se, 109.7 mg Zn, 6,770 IU Vit. A, 1590 IU Vit. D, 40 IU Vit. E

[3]Calculated from the average DMI of 17.8 kg/d

Ad libitum dry matter intake (DMI) was measured for 4 d prior to the initiation of the experiment, and cows were restricted to that level of intake for the duration of the study to avoid confounding effects of DMI and treatments. The cows were fed once daily except for the last 4 d of each

experimental period, during which time they were manually fed 6 equal meals every 4 h to maintain steady-state conditions.

2.2.3 Sampling

Feed amounts offered and refused were recorded daily to calculate DMI. Moisture content of ingredients was determined weekly; the rations were then adjusted to maintain constant delivery of DM. On the last 3 d of each experimental period, orts samples were collected and stored at −20°C until analyzed for DM content. Milk samples were collected on the last 3 d of each period from the AM and PM milkings.

On d 9 of each period, all cows received a 4-h (from 1030 to 1430 h) jugular infusion of D [6, 6-$^{2}H_2$] glucose (99 mole % excess (MPE); Cambridge Isotope Laboratories, Andover, MA). The labeled glucose was continuously infused at a constant rate of 23.5 mmol/h by syringe pump. Blood samples (n=5) were collected into heparinized tubes from the contralateral jugular vein, once before the infusion began and then every 30 min from 1230 to 1430 h. Samples were centrifuged for 25 min at 1,800 × g at 4°C and the harvested plasma was stored at -80°C until analysis.

On d 10 of each experimental period starting at 0430 h, six blood samples were collected every 2 h from a coccygeal vessel (arterial blood) and subcutaneous abdominal vein (venous blood) by venipuncture into sodium heparinized tubes. Samples were immediately placed on ice and centrifuged for 25 min at 1,800 × g at 4°C within 1 h of collection. Plasma was stored at −20°C for acetate, lactate, urea and BHBA analyses. An aliquot (1 g) of plasma was weighed and mixed with 200 µg of a [U-^{13}C] glucose solution (16 mM; 98 % MPE, Spectra Stable Isotopes Laboratories, Andover, MA) to determine glucose concentration and IE. For AA analysis, 1 g of

plasma was added to 200 µg of an internal standard of AA labeled with stable isotopes as described by Doepel et al. (2010). Samples were stored at -80ºC until subsequent analysis.

2.2.4 Laboratory Analyses

Ingredient samples were dried in a forced air oven at 55ºC for 72 h, ground through a Wiley mill No: 02 (1-mm screen; Arthur H. Thomas, Philadelphia, PA) and then pooled by period. They were sent to a commercial laboratory (Dairy One Inc, Ithaca, NY) for determination of CP, acid detergent fibre (ADF), neutral detergent fibre (NDF), calcium, magnesium, phosphorus, and potassium by wet chemistry. Milk samples were analyzed for protein, fat, lactose and milk urea nitrogen (MUN) by infrared spectroscopy at Central Milk Testing Laboratory (Edmonton, Alberta, Canada). Milk non-protein N level was analyzed with the micro-Kjeldahl method on pooled samples from the collection period. Non-protein N was obtained by precipitation with trichloroacetic acid, with a final concentration of 12%. Plasma acetate concentrations were determined by an enzymatic assay procedure using acetyl-CoA synthase and malate dehydrogenase (Sigma, Saint Quentin Fallavier, France) at INRA, Saint Gilles, France (Lemosquet et al., 2009a). Plasma lactate was measured using an enzymatic assay (LACT2, Cobas, Roche diagnosis GmbH D68298 Mannheim Indianapolis, IN, USA) at the Atlantic Veterinary College (Charlottetown, Prince Edward Island, Canada) and BHBA was measured using an Olympus AU680 Chemistry Analyzer (Veterinary Diagnostic Lab, University of Illinois, Urbana-Champaign, Urbana, IL). Blood urea-N (BUN) was determined calorimetrically (BUN No: 0580, Stanbio, Laboratory Boerne, Texas) with the end product measured at an absorbance of 520 nm. Plasma AA and glucose concentrations were determined by an isotope dilution method by GC-MS (Calder et al., 1999). For glucose, concentrations were analyzed by measuring ions 242 and 247

of the processed samples. Briefly, 100 μL of plasma were mixed with 300 μL of a 2:1 mixture of acetonitrile:ethanol for deproteinization and the derivatization was performed with penta-acetate, followed by addition of 50 μL of acetonitrile. Using GC-MS (GC 8090 coupled to MS 5973; Agilent Technologies, Wilmington, DE), ion mass was quantified in electron impact mode. Glucose IE was measured using ions 242 and 244, as described previously for concentrations, except that no internal standard was added. The derivatization was conducted with acetic anhydride pyridine and penta-acetate derivative, followed by addition of 50 μl of acetonitrile. The IE is expressed as MPE.

2.2.5 Calculations

Mammary plasma flow was calculated according to the Fick principle using Phe and Tyr as internal markers, with allowance for a 3.37% contribution from blood-borne proteins: MPF = [(milk Phe + Tyr) x 0.966]/ (AV difference Phe + Tyr) (Cant et al., 1993), using milk protein yield of the last experimental day. Milk AA output in protein synthesized in the MG was calculated using milk protein yield measured on the last day of each period, again with a 3.37% correction for blood-borne proteins, and AA composition proposed by Lapierre et al. (2012).

Nutrient uptake across the MG and fractional removal were calculated for each cow period as follows:

$$\text{Nutrient uptake} = AV_{diff} \text{ of the nutrient} \times \text{mammary plasma flow}$$

$$\text{Fractional removal} = \frac{AV_{diff} \text{ of the nutrient}}{\text{Arterial concentration of the nutrient}}$$

where AV_{diff} is the arterio-venous concentration difference.

The WBGRa was derived from the plasma IE as: $WBGRa = i \times (IE_{inf}/IE_{pl} - 1)$, where i is the infusion rate of the labeled tracer, IE_{inf} is the tracer enrichment of the infusate and IE_{pl} the tracer enrichment in plasma.

2.2.6 Statistical Analysis

Milk yield, milk composition and DMI were averaged over the last 3 d of each period. Metabolite concentrations and uptake data were averaged over the 6 sampling times for each cow for each period. Due to failure of abomasal catheter patency of 1 cow during the last 2 periods, samples for No-Thr and TAA treatments were collected for all five periods, whereas for No-Phe, No-Trp and CTL, samples were only obtained for 4 periods. All data were analyzed statistically using the MIXED procedure of SAS (SAS Institute Inc., 1999), with treatment as the fixed effect, and period and cow as random effects. Treatment differences were determined using pre-planned contrasts, comparing each treatment to TAA. Significance is declared at $P \leq 0.05$ and a tendency at $0.05 < P \leq 0.15$, and results are reported as least squares mean (LSM) with standard error of the mean (SEM).

2.3 Results

2.3.1 Dry Matter Intake and Milk Yield

Dry matter intake and milk data are shown in Table 2-3. Relative to TAA, DMI was not different for No-Thr, No-Trp and CTL; however, it tended to be lower for No-Phe ($P = 0.10$). Milk yield tended to decrease ($P \leq 0.15$) with the CTL, No-Phe and No-Thr groups compared to TAA. Milk protein yield was lower ($P = 0.01$) for CTL and No-Phe relative to TAA, tended to be lower ($P = 0.13$) for No-Thr relative to TAA, and was not affected ($P = 0.48$) by No-Trp. Milk protein

content was not affected by treatment. Milk lactose yield tended to decrease (P = 0.15) with CTL relative to TAA. Milk lactose content was increased (P ≤ 0.06) with No-Phe, No-Trp and No-Thr relative to TAA. Milk fat yield was not affected by treatment ($P > 0.15$). Fat content was higher in the No-Thr group compared to TAA ($P = 0.04$) and tended to be higher in No-Trp relative to TAA ($P = 0.11$). Milk urea N was lower for CTL than TAA ($P \leq 0.01$) but was not different among the other treatment groups.

Table 2-3 Effect of deletion of Phe, Thr or Trp from an amino acid (AA) infusion on dry matter intake (DMI) and milk production and composition

	Treatment[1]						P-value contrast[2]			
Item	CTL	No-Phe	No-Thr	No-Trp	TAA	SEM[3]	TAA CTL	TAA-No Phe	TAA-No Thr	TAA-No Trp
DMI, kg/d	17.8	17.0	17.6	17.2	18.0	0.60	0.69	0.10	0.46	0.19
Milk yield, kg/d	29.9	30.1	30.4	32.8	33.4	2.13	0.10	0.12	0.15	0.77
Fat, g/d	1038	1077	1156	1246	1123	0.07	0.34	0.60	0.71	0.18
Crude protein, g/d	781	771	843	883	914	0.04	0.01	0.01	0.13	0.48
Lactose, g/d	1360	1381	1391	1506	1502	0.09	0.15	0.22	0.25	0.97
Milk composition										
Fat, %	3.58	3.59	3.92	3.80	3.41	0.23	0.44	0.43	0.04	0.11
Crude protein, %	2.63	2.57	2.83	2.70	2.75	0.13	0.37	0.21	0.55	0.68
Lactose, %	4.55	4.60	4.59	4.60	4.50	0.05	0.27	0.05	0.06	0.04
MUN[4], mg/dL	8.81	13.54	14.63	14.24	14.12	0.94	0.01	0.65	0.70	0.93

[1]CTL = water; TAA = total amino acids; No-Phe = TAA without Phe; No-Thr = TAA without Thr; No-Trp = TAA without Trp;
[2]*P*-value for contrasts (comparing TAA to each treatment)
[3]SEM (standard error of the mean) given for n = 4
[4]MUN = milk urea-N

2.3.2 Energy Yielding Nutrients

Relative to TAA, plasma concentrations of BHBA, glucose and lactate were not affected by CTL or by the deletion from the AA mixture of a single AA (Table 2-4). Respectively, their average values were 0.51 ± 0.04, 3.47 ± 0.14 and 0.54 ± 0.13 mM. Acetate concentration was decreased in No-Thr ($P = 0.05$) relative to TAA but the other treatments were not different from TAA ($P \geq 0.29$). The AV difference for acetate and BHBA tended ($P \leq 0.08$) to be lower for No-Thr compared to TAA. Glucose AV difference was decreased ($P \leq 0.03$) in No-Phe and No-Thr relative to TAA, and lactate AV difference tended to be lower ($P = 0.07$) for No-Trp compared with TAA.

Mammary uptake of acetate was higher ($P = 0.05$) for No-Trp relative to TAA (Table 2.4). Mammary uptake of the other energy metabolites was not affected by treatment. Average mammary uptake of BHBA, glucose and lactate was 113 ± 15.0, 432 ± 43.0 and 127 ± 46.4 mmol/h, respectively. Glucose fractional removal was lower ($P \leq 0.01$) for No-Phe and No-Thr compared to TAA. For BHBA fractional removal tended to decrease with No-Thr compared to TAA ($P = 0.15$). Lactate fractional removal was lower ($P = 0.01$) in No-Trp compared to TAA.

Table 2-4 Effect of deletion of Phe, Thr or Trp from an amino acid (AA) infusion on arterial concentration, arterio-venous (AV) difference, mammary uptake and mammary fractional removal of plasma metabolites

Parameter	Treatment[1]					SEM[3]	*P*-value contrast[2]			
	CTL	No-Phe	No-Thr	No-Trp	TAA		TAA-CTL	TAA-No Phe	TAA-No Thr	TAA-No Trp
Arterial concentration, mM										
Acetate	1.69	1.49	1.26	1.69	1.54	0.13	0.29	0.73	0.05	0.31
BHBA[4]	0.54	0.49	0.46	0.53	0.53	0.04	0.76	0.43	0.19	1.00
Glucose	3.34	3.55	3.53	3.46	3.46	0.14	0.23	0.33	0.38	0.99
Lactate	0.71	0.52	0.62	0.35	0.52	0.13	0.18	0.99	0.43	0.21
AV difference, mM										
Acetate	1.08	0.95	0.80	1.14	1.02	0.11	0.64	0.54	0.08	0.35
BHBA[4]	0.20	0.18	0.16	0.21	0.22	0.02	0.60	0.30	0.07	0.75
Glucose	0.75	0.67	0.62	0.78	0.84	0.06	0.19	0.03	0.01	0.41
Lactate	0.25	0.21	0.27	0.07	0.26	0.08	0.89	0.64	0.89	0.07
Mammary uptake, mmol/h										
Acetate	546	599	622	706	537	84.7	0.91	0.42	0.24	0.05
BHBA[4]	98	112	123	118	115	15.0	0.33	0.88	0.59	0.82
Glucose	368	411	477	463	441	43.0	0.18	0.56	0.45	0.66
Lactate	126	124	204	48	135	46.4	0.87	0.84	0.22	0.16
Mammary fractional removal, %										
Acetate	64.1	63.7	62.8	67.7	66.1	2.20	0.51	0.44	0.26	0.59
BHBA[4]	37.1	37.5	34.2	39.2	40.7	3.38	0.43	0.48	0.15	0.74
Glucose	21.9	18.8	17.7	22.4	24.3	1.56	0.23	0.01	0.01	0.33
Lactate	35.0	39.2	44.3	18.4	48.1	8.08	0.14	0.30	0.62	0.01

[1]CTL= water; TAA = total amino acids; No-Phe = TAA without Phe; No-Thr = TAA without Thr; No-Trp = TAA without Trp
[2]*P*-value for contrasts (comparing TAA to each treatment)
[3]SEM (standard error of the mean) given for n = 4
[4]BHBA= Beta-hydroxybutyrate

2.3.3 Whole Body Glucose Rate of Appearance

Whole-body glucose rate of appearance in the No-Thr treatment was reduced (P = 0.06) relative to TAA (Table 2-5). It was not different for the other treatment groups relative to TAA. Isotopic enrichment was increased in No-Thr (P = 0.06) and was not affected in other groups relative to TAA. The milk lactose output:glucose WBGRa ratio was not different among treatments and averaged 0.54. Milk lactose output as a percentage of glucose mammary uptake was lower for No-Thr compared to TAA, whereas the other treatments did not differ from TAA.

Table 2-5 Effect of deletion of Phe, Thr or Trp from an amino acid (AA) infusion on glucose isotopic enrichment (MPE, %*) and whole body glucose rate of appearance (mmol/h)

Item	Treatments[1]					SEM[3]	*P*-value contrast[2]			
	CTL	No-Phe	No-Thr	No-Trp	TAA		TAA -CTL	TAA -No Phe	TAA -No Thr	TAA -No Trp
IE[4]	3.79	3.52	4.06	3.74	3.53	0.27	0.35	0.96	0.06	0.46
WBGRa[5]	597.8	642.2	575.8	599.2	645.5	38.1	0.21	0.93	0.06	0.22
L/W[6]	53	52	53	58	55	4.0	0.57	0.49	0.66	0.63
L/G[7]	84	78	65	75	80	6.1	0.67	0.65	0.05	0.73

[1]CTL = water; TAA = total amino acids; No-Phe = TAA without Phe; No-Thr = TAA without Thr; No-Trp = TAA without Trp
[2]*P*-value for contrasts (comparing TAA to each treatment)
[3]SEM (standard error of the mean) given for n = 4
[4]IE = Isotopic enrichment
[5]WBGRa = Whole body glucose rate of appearance
[6]L/W = Lactose/WBRa, %
[7]L/G = Lactose/glucose MG flux, %
*MPE = Mole percent excess

2.3.4 Arterial Concentrations of Amino Acids and Urea

Amino acid arterial concentrations are shown in Table 2-6. Except for Lys, arterial concentrations of EAA decreased ($P \leq 0.05$) or tended to decrease (Trp: P = 0.09) in CTL compared with TAA. Concentrations of Phe, Thr and Trp decreased ($P \leq 0.01$) when the respective AA were deleted from the AA mixture relative to TAA. Leucine and Val concentrations tended to

increase ($P \leq 0.13$) with No-Phe compared with TAA, whereas Phe and Val concentrations tended to increase ($P \leq 0.15$) with No-Thr compared with TAA. Leucine, Phe and Val concentrations tended to increase ($P \leq 0.08$) in No-Trp relative to TAA.

For the NEAA, arterial concentrations of Gln, Pro and Tyr were lower with CTL than TAA whereas Glu ($P = 0.05$) concentration was higher. With No-Phe, the concentration of Ser tended to increase ($P = 0.13$) relative to TAA. With the No-Thr infusion, arterial concentration of Cys was lower compared with TAA ($P < 0.01$) whereas Ser ($P = 0.03$) concentration increased. Concentrations of Gly and Ser were higher ($P \leq 0.01$) with No-Trp compared with TAA, whereas Cys ($P = 0.01$) was lower and Gln and Glu tended to be lower ($0.05 < P \leq 0.15$) relative to TAA.

Urea concentration was lower with CTL ($P = 0.01$) relative to TAA and higher with No-Phe ($P = 0.04$) compared with TAA.

Table 2-6 Effect of deletion of Phe, Thr or Trp from an amino acid (AA) infusion on arterial concentrations of AA and urea-N.

Item	Treatments[1]					SEM[3]	*P*-value contrast[2]			
	CTL	No-Phe	No-Thr	No-Trp	TAA		TAA-CTL	TAA-No Phe	TAA-No Thr	TAA-No Trp
AA, µM										
Alanine	193.0	173.0	169.4	173.7	181.2	12.30	0.43	0.58	0.43	0.61
Asparagine	31.1	37.5	37.2	38.1	37.7	2.64	0.08	0.97	0.88	0.89
Aspartate	25.1	18.4	19.0	17.8	18.4	4.25	0.18	0.99	0.89	0.89
Cysteine	104.4	104.8	91.5	83.5	110.8	6.00	0.19	0.22	<0.01	<0.01
Glutamine	183.0	252.8	247.4	215.5	243.5	15.34	<0.01	0.50	0.75	0.07
Glutamate	69.0	63.8	59.3	56.2	61.4	4.33	0.05	0.50	0.52	0.15
Glycine	211.3	211.8	207.9	239.2	192.1	25.18	0.40	0.39	0.45	0.08
Histidine	22.7	59.9	59.5	55.3	62.3	4.62	<0.01	0.66	0.58	0.21
Isoleucine	93.1	133.4	119.3	131.6	118.9	7.67	0.02	0.16	0.96	0.21
Leucine	113.0	213.5	201.3	218.3	186.5	12.24	<0.01	0.13	0.35	0.08
Lysine	57.7	68.8	64.1	70.2	63.7	6.03	0.44	0.51	0.96	0.41
Methionine	26.6	31.3	32.1	31.9	32.8	2.13	0.04	0.57	0.77	0.72
Phenylalanine	46.6	21.1	57.8	58.3	54.0	2.66	0.01	<0.01	0.09	0.08
Proline	76.7	192.6	186.9	182.3	178.7	17.04	<0.01	0.24	0.44	0.75
Serine	74.5	104.1	113.8	124.7	84.0	12.88	0.45	0.13	0.03	0.01
Threonine	72.1	113.9	22.9	108.7	106.0	8.19	0.01	0.44	<0.01	0.78
Tryptophan	38.2	45.7	44.5	13.4	42.4	2.77	0.09	0.17	0.33	<0.01
Tyrosine	45.5	69.8	75.7	75.8	71.5	3.91	<0.01	0.67	0.28	0.30
Valine	171.0	312.1	297.2	311.4	264.8	16.65	<0.01	0.06	0.15	0.06
Urea-N, mM	6.8	13.2	11.6	11.6	11.2	0.74	0.01	0.04	0.62	0.62

[1]CTL = water; TAA = total amino acids; No-Phe = TAA without Phe; No-Thr = TAA without Thr; No-Trp = TAA without Trp
[2]*P*-value for contrasts (comparing TAA to each treatment)
[3]SEM (standard error of the mean) given for n = 4

2.3.5 Mammary Plasma Flow and Mammary AA Uptake

Table 2-7 shows MPF and mammary uptake of AA. Mammary plasma flow was higher with the No-Thr infusion relative to TAA ($P = 0.01$); however it did not differ with No-Phe, No Trp and CTL treatments compared with TAA.

Mammary uptake of His, Ile, Leu, Phe, Tyr, Val, Group-1 AA, Group-2 AA, total EAA-N (TEAA-N) and BCAA was lower ($P \leq 0.04$) with the CTL relative to TAA. Mammary uptake of Met, Pro, total AA-N (TAA-N) and Thr tended to be lower with CTL compared with TAA. Mammary uptake of Asp tended to be higher ($P = 0.07$) with CTL relative to TAA.

Mammary uptakes of Tyr, Phe, Met and Gln were reduced ($P \leq 0.05$) with No-Phe compared to TAA whereas uptake of Cys was higher ($P \leq 0.03$) relative to TAA. Mammary uptake of Asn, Ser, Group-1 AA, total NEAA-N (TNEAA-N) and TAA-N tended to be lower ($0.05 < P \leq 0.15$) with No-Phe relative to TAA.

When No-Thr was infused mammary uptakes of Asn, Pro, Ser, and Tyr were lower ($P \leq 0.05$) relative to TAA. Mammary uptake of Glu ($P \leq 0.03$) was higher with No-Thr infusion relative to TAA, and tended to be higher for Asp and Cys ($0.05 < P \leq 0.15$). Mammary uptake of TNEAA-N tended to decrease ($P \leq 0.09$) with No-Phe and No-Thr relative to TAA. The No-Trp treatment had no effect on mammary uptake of any AA.

Table 2-7 Effect of deletion of Phe, Thr or Trp from an amino acid (AA) infusion on mammary AA uptake (mmol/h) and plasma flow

Item	Treatments[1]					SEM[3]	*P*-value contrast[2]			
	CTL	No-Phe	No-Thr	No-Trp	TAA		TAA-CTL	TAA-No Phe	TAA-No Thr	TAA-No Trp
Plasma flow, L/h	497	601	775	603	524	52.6	0.68	0.24	<0.01	0.23
Asparagine	5.3	4.6	4.3	5.8	6.1	0.75	0.28	0.06	0.03	0.69
Aspartate	4.8	3.2	4.4	3.7	2.3	1.03	0.07	0.48	0.09	0.30
Cysteine	2.2	3.0	2.4	1.9	0.9	0.62	0.18	0.03	0.10	0.30
Glutamine	26.7	22.8	27.7	25.7	30.1	2.74	0.32	0.05	0.44	0.21
Glutamate	20.5	20.2	22.0	18.6	17.8	2.21	0.19	0.23	0.03	0.66
Glycine	4.1	1.5	- 2.2	-1.0	1.9	2.22	0.23	0.85	0.03	0.18
Histidine	5.8	6.2	6.6	7.0	7.0	0.50	0.04	0.17	0.35	0.99
Isoleucine	16.6	20.7	23.7	23.4	22.1	1.19	<0.01	0.35	0.25	0.38
Leucine	25.5	33.3	37.3	37.5	34.6	1.79	<0.01	0.52	0.16	0.16
Lysine	19.5	22.6	25.8	26.2	22.2	3.11	0.52	0.92	0.36	0.34
Methionine	6.2	6.0	6.4	7.0	6.9	0.47	0.12	0.04	0.23	0.93
Phenylalanine	10.1	9.0	10.9	11.9	11.8	0.85	0.04	<0.01	0.22	0.92
Proline	7.7	9.1	6.2	10.9	10.5	1.61	0.10	0.39	0.01	0.77
Serine	6.7	4.9	2.7	8.1	9.3	2.56	0.23	0.06	0.01	0.55
Threonine	12.1	12.6	12.6	13.6	14.2	1.06	0.10	0.20	0.18	0.63
Tryptophan	2.9	3.0	3.1	2.9	3.1	0.39	0.64	0.81	0.92	0.62
Tyrosine	8.9	9.3	8.7	10.0	10.6	0.60	0.02	0.05	0.01	0.36
Valine	21.5	28.7	29.3	28.0	29.0	1.15	<0.01	0.78	0.82	0.34
Group 1 AA-N[4]	48.5	49.0	51.9	55.9	56.8	3.77	0.04	0.06	0.17	0.82
Group 2 AA-N[5]	101.7	126.8	141.8	140.9	130.1	8.85	0.02	0.76	0.26	0.33
TEAA-N[6]	152.7	178.6	197.6	199.9	190.5	12.28	0.02	0.41	0.59	0.51
TNEAA-N[7]	132.4	112.1	116.1	129.2	139.4	13.31	0.58	0.06	0.09	0.46
BCAA[8]	63.7	82.8	90.2	88.5	85.7	3.74	<0.01	0.46	0.21	0.46
TAA-N[9]	284.0	289.4	321.4	337.5	334.1	22.34	0.08	0.12	0.63	0.90

[1]CTL = water; TAA= total amino acids; No-Phe = TAA without Phe; No-Thr = TAA without Thr; No-Trp = TAA without Trp; [2]P-value for contrasts (comparing TAA to each treatment); [3]SEM (standard error of the mean) given for n = 4; [4]Group 1 AA-N = His, Met, Phe, Trp, and Tyr; [5]Group 2 AA-N = Ile, Leu, Lys, and Val; [6]TEAA-N (total essential AA-N) = His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; [7]TNEAA-N (total non- essential AA-N) = Ala, Asn, Asp, Cys, Glu, Gln, Gly, Pro, Ser, Tyr; [8]BCAA = Ile, Leu, Val; [9]TAA-N (total AA-N) = Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val

2.3.6 Mammary AA Uptake:Milk Protein Output Ratio

Mammary uptake:milk protein output ratios are shown in Table 2-8. The ratio for Leu, Val and the BCAA tended to be lower ($P \leq 0.14$) with CTL than TAA. The ratio for Asp and Glu was higher ($P \leq 0.05$) for CTL compared to TAA, and tended to be higher ($P \leq 0.15$) for Cys and TNEAA-N. When No-Phe was infused, the uptake:output ratio increased ($P \leq 0.05$) for Cys, Glu, Ile, Leu, Group 2 AA, BCAA and TEAA-N and tended to increase for Lys and Val ($P \leq 0.13$) relative to TAA.

The No-Thr treatment resulted in an increment ($P \leq 0.05$) of the mammary uptake:output ratio for Asn, Glu, Ile, Leu, Lys, Val, BCAA, Group 2 AA, and TNEAA-N relative to TAA. With No-Thr compared with TAA, the ratio tended to increase ($P \leq 0.10$) for Asp and Cys, tended to decrease ($P \leq 0.08$) for Ala, and decreased ($P \leq 0.03$) for Pro and Ser. With the No-Trp infusion the uptake:output ratio tended to increase for Leu ($P \leq 0.13$) relative to TAA.

Table 2-8 Effect of deletion of Phe, Thr or Trp from an amino acid (AA) infusion on mammary AA uptake:milk protein output ratio

	Treatments[1]						*P*-value contrast[2]			
AA	CTL	No-Phe	No-Thr	No-Trp	TAA	SEM[3]	TAA-CTL	TAA-No Phe	TAA-No Thr	TAA-No Trp
Alanine	1.19	0.58	0.18*	0.62	0.77	0.33	0.21	0.54	0.08	0.62
Asparagine	0.55*	0.47*	0.40*	0.51*	0.53*	0.05	0.79	0.34	0.05	0.75
Aspartate	0.56*	0.38*	0.50*	0.37*	0.25*	0.11	0.05	0.36	0.09	0.39
Cysteine	1.13†	1.68*	1.18	0.83	0.51†	0.29	0.15	0.02	0.10	0.43
Glutamine	1.34*	1.17	1.39*	1.09	1.31*	0.15	0.85	0.40	0.63	0.20
Glutamate	0.80*	0.78*	0.83*	0.61*	0.58*	0.08	0.01	0.02	<0.01	0.66
Glycine	0.53†	0.22*	-0.34*	-0.13*	0.21*	0.28	0.17	0.98	0.02	0.18
Histidine	1.05	1.16*	1.15*	1.08†	1.08	0.05	0.62	0.23	0.28	0.97
Isoleucine	1.22*	1.51*	1.67*	1.41*	1.33*	0.09	0.16	0.05	<0.01	0.38
Leucine	1.13†	1.47*	1.58*	1.37*	1.26*	0.07	0.09	0.01	<0.01	0.13
Lysine	1.12	1.32*	1.43*	1.28*	1.04	0.13	0.62	0.13	0.04	0.19
Methionine	1.03	1.00	1.03	0.98	0.97	0.03	0.22	0.52	0.16	0.89
Phenylalanine	1.07*	0.97	1.13*	1.08*	1.07*	0.03	0.96	<0.01	0.07	0.70
Proline	0.28*	0.34*	0.20*	0.35*	0.32*	0.05	0.41	0.75	0.03	0.65
Serine	0.36*	0.27*	0.12*	0.38*	0.39*	0.12	0.68	0.16	0.01	0.84
Threonine	1.05	1.13*	1.05	1.01	1.05	0.04	0.89	0.16	0.99	0.54
Tryptophan	1.27*	1.35*	1.30*	1.09	1.17	0.13	0.55	0.31	0.42	0.66
Tyrosine	0.94*	1.00	0.88*	0.90*	0.93*	0.02	0.68	0.04	0.06	0.28
Valine	1.24†	1.66*	1.67*	1.36*	1.43*	0.11	0.14	0.07	0.05	0.56
Group 1 AA-N[4]	1.05	1.08*	1.09*	1.03	1.04	0.03	0.85	0.35	0.30	0.81
Group 2 AA-N[5]	1.13†	1.44*	1.55*	1.33*	1.22*	0.08	0.41	0.04	<0.01	0.27
TEAA-N[6]	1.13	1.34*	1.40*	1.25	1.16	0.06	0.66	0.03	0.01	0.27
TNEAA-N[7]	0.78*	0.67*	0.62*	0.65*	0.69*	0.05	0.14	0.69	0.21	0.54
BCAA[8]	1.19*	1.54*	1.63*	1.37*	1.33*	0.08	0.09	0.02	<0.01	0.60
TAA-N[9]	0.93*	0.96	0.95†	0.95	0.91*	0.03	0.72	0.20	0.32	0.40

[1]CTL = water; TAA = total amino acids; No-Phe = TAA without Phe; No-Thr = TAA without Thr; No-Trp = TAA without Trp

[2]*P*-value for contrasts (comparing TAA to each treatment)

[3]SEM (standard error of the mean) given for n = 4

[4]Group 1 AA-N = His, Met, Phe, Trp, and Tyr

[5]Group 2 AA-N = Ile, Leu, Lys, and Val

[6]TEAA-N (total essential AA-N) = His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val

[7]TNEAA-N (total non- essential AA-N) = Ala, Asn, Asp, Cys, Glu, Gln, Gly, Pro, Ser, Tyr

[8]BCAA = Ile, Leu, Val

[9]TAA-N (total AA-N) = Ala, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val

*Different from 1 at $P \leq 0.05$; †different from 1 at $0.05 < P \leq 0.15$

2.3.7 Mammary Gland Fractional Removal of AA

Mammary gland fractional removal values are shown in Table 2-9. For Gln, His, Leu, Pro, Thr, Tyr, Val, Group 1 AA, Group 2 AA, TEAA-N, TNEAA-N, BCAA, and TAA-N, fractional removal was increased ($P \leq 0.05$) with CTL infusion relative to TAA. The fractional removal of Cys, Met and BCAA tended to increase ($0.05 < P \leq 0.15$) with CTL relative to TAA infusion.

When No-Phe was infused, the MG fractional removal was lower ($P \leq 0.05$) for Asn, Gln, Ile, Leu, Lys, Met, Val, Group 2 AA, TEAA-N, TNEAA-N, BCAA, and TAA-N compared with TAA. Fractional removal of Ala, Glu, Ser, Thr, Tyr tended to decrease ($0.05 < P \leq 0.15$) with No-Phe infusion relative to TAA treatment; however for Cys and Phe, it was higher ($P \leq 0.05$) relative to TAA when No-Phe was infused.

Mammary gland fractional removal of Thr was increased ($P \leq 0.01$) with No-Thr infusion relative to TAA. For Tyr, mammary fractional removal tended to decrease ($0.05 < P \leq 0.15$) relative to TAA. Mammary fractional removal of Asp, Cys, and His, was not changed when No – Thr was infused relative to TAA. For all other NEAA and EAA mammary fractional removal was decreased ($P \leq 0.05$) relative to TAA with No-Thr treatment.

Mammary fractional removal was decreased ($P \leq 0.05$) for Gln, Val, TNEAA-N and TAA group relative to TAA when No-Trp infused. However mammary fractional removal tended to decrease ($0.05 < P \leq 0.15$) in Asn, Group 2 AA and BCAA compared with TAA. Mammary fractional removal of Trp was increased ($P \leq 0.01$) with No-Trp infusion relative to TAA.

Table 2-9 Effect of deletion of Phe, Thr or Trp from an amino acid (AA) infusion on mammary fractional removal of AA (%)

AA	Treatments[1]					SEM[3]	*P*-value contrast[2]			
	CTL	No-Phe	No-Thr	No-Trp	TAA		TAA-CTL	TAA-No Phe	TAA-No Thr	TAA-No Trp
Alanine	13.5	6.3	2.3	7.2	12.4	3.88	0.78	0.14	0.03	0.20
Asparagine	33.8	20.3	14.6	25.5	30.7	2.56	0.32	0.01	<0.01	0.11
Aspartate	34.5	27.6	28.8	31.9	27.3	3.83	0.18	0.94	0.74	0.37
Cysteine	4.3	5.0	3.3	3.7	1.9	1.02	0.11	0.05	0.31	0.23
Glutamine	29.1	15.6	14.5	19.5	23.8	1.33	<0.01	<0.01	<0.01	0.01
Glutamate	59.2	50.9	48.1	56.0	55.3	2.26	0.17	0.12	0.01	0.82
Glycine	4.0	2.2	-1.5	-0.5	2.2	1.88	0.28	1.00	0.03	0.14
Histidine	60.8	18.2	14.6	21.8	21.7	4.64	<0.01	0.59	0.25	0.99
Isoleucine	36.9	26.9	25.9	31.5	35.7	2.98	0.75	0.04	0.02	0.26
Leucine	46.1	26.9	24.1	30.4	35.7	2.54	0.01	0.02	<0.01	0.13
Lysine	67.2	54.2	51.6	62.1	65.8	2.90	0.67	<0.01	<0.01	0.26
Methionine	46.5	31.6	26.3	37.0	40.6	3.10	0.14	0.04	<0.01	0.36
Phenylalanine	43.0	72.1	24.2	35.3	42.3	3.91	0.89	<0.01	<0.01	0.18
Proline	22.5	8.6	4.1	10.7	11.5	1.74	<0.01	0.22	0.01	0.71
Serine	21.2	10.2	1.0	12.7	20.5	6.71	0.91	0.09	0.01	0.18
Threonine	33.8	19.5	70.6	21.9	25.8	2.61	0.04	0.09	<0.01	0.28
Tryptophan	15.7	11.5	9.0	37.4	14.3	2.19	0.64	0.36	0.09	<0.01
Tyrosine	40.6	22.7	15.1	23.8	28.4	3.14	0.01	0.12	<0.01	0.20
Valine	25.4	15.7	12.9	15.9	21.1	1.54	0.05	0.02	<0.01	0.03
Group 1 AA-N[4]	38.0	21.3	15.5	26.6	25.2	2.00	<0.01	0.18	<0.01	0.62
Group 2 AA-N[5]	42.4	27.4	24.7	30.5	35.5	2.01	0.03	0.01	<0.01	0.09
TEAA-N[6]	40.1	25.0	22.7	29.0	31.1	1.73	<0.01	0.03	<0.01	0.38
TNEAA-N[7]	21.7	12.8	9.7	13.6	18.1	1.40	0.03	0.01	<0.01	0.02
BCAA[8]	34.4	21.6	19.1	23.8	28.9	2.06	0.06	0.02	<0.01	0.08
TAAN[9]	28.8	18.2	15.9	20.1	24.0	1.19	0.01	<0.01	<0.01	0.04

[1]CTL = water; TAA = total amino acids; No-Phe = TAA without Phe; No-Thr = TAA without Thr; No-Trp = TAA without Trp; [2]P-value for contrasts (comparing TAA to each treatment); [3]SEM (standard error of the mean) given for n = 4; [4]Group 1 AA-N = His, Met, Phe, Trp, and Tyr; [5]Group 2 AA-N = Ile, Leu, Lys, and Val; [6]TEAA-N (total essential AA-N) = His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val; [7]TNEAA-N (total non- essential AA-N) = Ala, Asn, Asp, Cys, Glu, Gln, Gly, Pro, Ser, Tyr;[8]BCAA = Ile, Leu, Val, [9]TAA-N (total AA-N) = all AA

2.4 Discussion

2.4.1 Dry Matter Intake

Treatments did not affect DMI except for No-Phe, for which DMI tended to decrease relative to TAA. This reduction may be due to a severe imbalance of AA supply when Phe was removed from the infusate. A reduction in feed intake may occur through suppression of the appetite centre in the brain, due to insufficient individual EAA supply (Harper et al., 1970), but it is not clear why in the current study this effect reached significance only for the No-Phe treatment. Perhaps the Phe limitation was stronger than limitations imposed when Thr and Trp were removed from the infusate. Lapierre et al. (2009) reported no DMI change with deletion of Lys from an AA mixture infused in lactating cows. Doepel and Lapierre (2011) also reported no change in DMI when Arg was depleted from an abomasal infusion of AA. However, in the study of Weekes et al. (2006), in which cows were infused with AA mixtures and fed ad libitum, there were transient reductions in DMI with the infusions devoid of Lys or Met or His relative to the negative control, but as the infusion period continued (d 4 to 6 of the infusion) these differences in DMI disappeared. The reduction in DMI in the Weekes et al. (2006) study was probably due to a more severe imbalance of AA, as the CP concentration of the basal diet was only 9.3%.

2.4.2 Milk Yield and Composition and Energy-Yielding Nutrients

With the exception of No-Trp, the AA deletion treatments tended to reduce milk yield relative to TAA, indicating that AA, either as a composite or individually, were not supplied in sufficient amounts to sustain milk synthesis. Although EAA and NEAA were both deficient in the CTL treatment, the effect on milk yield was likely due to the supply of EAA, because total NEAA-N mammary uptake was unaffected by CTL relative to TAA (Table 2.7). This is supported by the

work of Doepel and Lapierre (2010) who reported that the addition of NEAA to an EAA infusion did not improve milk yield. The effect of Phe and Thr deficiencies was particularly evident in the milk protein yield response. The MG responded to a deficiency of Phe and Thr by reducing milk protein secretion through different mechanisms. With No-Phe, Phe uptake decreased mainly through a reduction of A-V difference, as MPF only numerically increased. With No-Thr, MPF increased by 32%, and despite a large decreased A-V difference, Thr uptake only numerically (13%) decreased. Tryptophan had no effect on milk protein yield, suggesting that even with its deletion, it was supplied in sufficient amounts to meet the cows' needs with the low MP diet. Milk protein content did not differ among treatments, in contrast to Doelman et al. (2013) who reported that protein concentration decreased by 0.36, 0.37 and 0.25 percentage points when Met, Phe, and His, respectively, were removed from an EAA infusion.

Milk lactose content increased with the individual AA deletions relative to TAA. This result was in agreement with the study of Doepel and Lapierre (2010) in which removal of EAA from an AA abomasal infusion increased milk lactose percentage. Milk lactose yield also decreased with removal of the EAA in that study, whereas in the current study, lactose yield was unaffected by deletion of individual AA. The lack of an effect on lactose yield coincided with glucose mammary uptake, which was not different between treatments, and WBGRa, which was only different between TAA and No-Thr. Contrary to that found with the individual AA deletions, lactose yield tended to be lower with CTL than with TAA, in agreement with data presented by Lapierre et al. (2010) that post-ruminal infusions of casein or AA with a casein profile consistently resulted in a positive milk lactose yield response.

Milk fat yields of the CTL, No-Phe and No-Thr treatments were not different from TAA, consistent with a lack of response to treatment of the mammary uptake of acetate. The fat yield of No-Trp was numerically the highest and was associated with an increase in acetate mammary uptake. Milk yield tended to be lower with CTL, No-Phe, and No-Thr without a reduction in mammary uptake of BHBA, glucose or lactate. Although milk yield tended to be lower, milk lactose yield and total solids yield (sum of fat, protein, and lactose) were not different among treatments except for CTL, which tended to be lower than TAA. These observations were in alignment with the lack of energetic substrate uptake differences, except for a tendency for reduced glucose uptake for the CTL treatment relative to TAA. Indeed, BHBA and lactate were summed to represent total energy supply to the MG and uptake of this energy variable was also not different among treatments.

2.4.3 Whole Body Glucose Rate of Appearance

With the exception of No-Thr, glucose WBGRa was not significantly different among treatments; however, CTL and No-Trp were numerically lower than TAA. Usually, WBGRa increases with casein or TAA infusion, concurrent with an increase in milk lactose yield (e.g. Lemosquet et al., 2009b; Galindo et al., 2011), in line with the comparison between CTL and TAA.

The variation in the responses to deletion of Phe, Thr and Trp on WBGRa is difficult to explain. Theoretically glucose precursor availability should increase with increased AA supply and no increment in milk protein secretion. One hypothesis would be that WBGRa is driven as much by glucose precursor availability as it is from metabolic demand, especially for AA. Indeed, when comparing isoenergetic infusions of glucose or glucose precursors, the WBGRa increment observed with a NEAA infusion was only 50% of the increment observed with a glucose infusion

(Lemosquet et al., 2009a). Although no research has been conducted to determine the WBGRa changes with a limited supply of individual AA in dairy cows, Weekes et al. (2006) observed an increment in glucose plasma concentrations when His, Lys or Met were deleted from an AA infusion mixture, with a corresponding decrease in milk protein yield. In the current study, there was no clear relationship between mammary glucose uptake or milk lactose yield and WBGRa. Milk lactose synthesis is most probably influenced by other unknown mechanisms in addition to glucose availability and mammary glucose uptake. In the current study, the lactose to mammary glucose uptake ratio was decreased with No-Thr, but it is interesting that the lactose to WBGRa ratio did not change among treatments. It appears that changes of either WBGRa or changes in mammary glucose uptake do not entirely influence lactose synthesis.

2.4.4 Urea and Amino Acid Metabolism

2.4.4.1 Arterial Concentrations

Urea-N concentration was drastically reduced with CTL compared to TAA, indicating a more efficient usage of AA at low MP supply. The TAA treatment supplied an additional 925 g of AA, but only resulted in an increase of 133 g of milk protein, resulting in a marginal efficiency of 14%. The AA profile of TAA was the same as that of casein; although this was reflective of milk composition, it may not supply the ideal balance of AA, considering that the mammary uptake to milk protein output ratio is not 1:1 for all AA. With No-Phe, urea-N was higher than with TAA, probably linked with the reduced milk protein secretion, leading to deamination of a larger quantity of AA. Weekes et al. (2006) also reported an increase in urea concentration when individual AA were removed from a complete AA infusion.

The decrease in EAA arterial concentrations, with the exception of Lys, with CTL is an indication that the EAA were deficient relative to the cows' requirements; this was expected considering that the diet was formulated to meet only 66% of the cows' MP requirement. Although Lys is considered the first limiting AA on corn-based diets (NRC, 2001), in this study it may have been supplied in sufficient amounts from the basal diet, which was based on alfalfa hay and barley silage. Phenylalanine concentration decreased as expected with No-Phe, but increased with No-Thr and No-Trp relative to TAA. For the No-Thr treatment, this increase likely reflects excess supply, since milk and milk protein yields were decreased, but the increase is difficult to explain with the No-Trp treatment, considering that milk protein output and Phe mammary uptake were similar between No-Trp and TAA. The concentration of Val tended to increase with the individual AA deletion treatments whereas the concentrations of the two branched-chain AA numerically increased with the deletion of Phe and Trp. Similar to Phe, these increases are associated with the reduced milk protein yield with the No-Phe and No-Thr treatments, but are harder to explain with No-Trp.

Glutamate and Gln concentrations tended to decrease with No-Trp compared with TAA even though milk protein yield and mammary uptake of Glu and Gln were not different between the treatments. This contrasts with the study of Weekes et al. (2006), in which deletion of Met, Lys, or His from an AA infusion reduced milk protein yield but had no effect on Glu and Gln concentrations.

2.4.4.2 Mammary Plasma Flow and Amino Acid Uptake

The increase in mammary plasma flow with No-Thr compared to TAA is consistent with previous AA deletion studies. Bequette et al. (2000) reported a 33% increase in mammary plasma

flow in goats when a His deficiency was imposed and Cant et al. (2003) proposed that removal of individual AA would increase mammary flow. Although the MG compensates for reduced AA availability by increasing MPF, that MPF was only numerically increased with No-Phe and No-Trp relative to TAA suggests that other factors are also involved in the regulation. Interestingly, a global reduction in AA availability, as seen with CTL, did not elicit an increment in MPF, as suggested from a meta-analysis (Lapierre et al., 2012).

With the exception of Asp, mammary uptakes of all AA with CTL were either lower or the same as TAA. This is an expected finding, and reflects the overall deficiency in supply of AA to the MG, which led to a reduction in milk yield and milk protein yield. With the single AA deletion treatments, the only decreases in individual EAA uptake were for Met and Phe with No-Phe. This was similar to the findings of Doepel and Lapierre (2011) in which deletion of Arg from a complete AA infusion only reduced the uptake of Arg. However, when grouped together, the uptake of Group 1 AA decreased or tended to decrease to accompany variations in milk protein yield. With No-Phe, although Phe arterial concentration was reduced by 61%, mammary uptake was reduced by only 24%, because fractional extraction increased by 71%, demonstrating the ability of the MG to adapt to variable substrate supply.

In terms of NEAA mammary uptake, there was no consistent response to single AA deletions. The No-Thr treatment invoked the greatest number of changes in NEAA uptake, but the responses were not consistent, with increases in uptake for some AA and decreases in others. The reasons for these inconsistencies are not known; with increased EAA uptake (particularly Group 2 AA) it can be predicted that NEAA uptake would decrease, because NEAA can be synthesized from EAA within the MG, but EAA uptake did not change with No-Thr. Overall, total NEAA

uptake decreased with both No-Phe and No-Thr compared to TAA, but this occurred without any change in EAA uptake. It is well established that the Group 2 AA can be converted to NEAA in the MG, and whereas uptake of the Group 2 AA did not change with No-Phe and No-Thr relative to TAA, the uptake:output ratio of these AA increased, indicating that the AA were used for purposes other than direct incorporation for milk protein synthesis. With No-Trp, there were no differences in AA uptake relative to TAA, which coincides with the lack of effect on milk and milk protein yield with this treatment.

2.5 Conclusion

Milk and milk protein yields were negatively impacted by deletion of Phe or Thr from a total AA infusion. This reduction in yield was accompanied by a marked reduction in mammary uptake of Phe with No-Phe, whereas a drastic increment in MPF led to only a numerical reduction of Thr uptake with Thr deletion. Milk lactose yield was not influenced by the deletion of single AA, consistent with the lack of effect of treatment on mammary glucose uptake.

Chapter Three: Overall Conclusion and Future Directions

3.1 Overall Conclusion

Deficiencies in Phe and Thr supply negatively affected milk and milk protein yields, whereas Trp deficiency did not affect these parameters. The elevated plasma urea-N concentration with No-Phe relative to TAA supported the reduced milk protein secretion, with the excess AA supply not used for milk protein synthesis converted to urea. Mammary plasma flow was increased with Thr deficiency. Mammary uptake of most EAA decreased with CTL relative to TAA. The decrease in plasma concentration of Phe and Thr with No-Phe and No-Thr infusion was accompanied by a reduction of milk protein yield, despite an increased mammary fractional removal. For No-Phe, the milk protein yield reduction was accompanied by a decreased mammary uptake of His, Met, Phe, Group 1 AA, TNEAA-N and TAA-N. For No-Thr, the more modest decrease of milk protein secretion was related to a reduction in mammary uptake of Group 1 AA (tendency), and TNEAA-N.

Despite a decreased mammary uptake of Group 1 AA with No-Phe (significant reduction) and No-Thr (numerical reduction), the uptake:output ratio maintained unity, indicating that Group 1 AA are exclusively used, on a net basis, for milk protein synthesis, irrespective of EAA limitation. Changes in utilization of various AA in the MG with a deficiency of Phe, Thr and Trp supply showed the metabolic flexibility of the MG when facing variable supply. The effect of Trp deficiency on mammary protein metabolism was negligible.

A limited supply of Thr and Trp increased milk fat concentration, but only No-Trp numerically increased milk fat yield. A limited supply of Phe, Thr, and Trp increased milk lactose concentration, but did not elevate milk lactose yield compared with TAA. Concentrations of

plasma metabolites related to energy metabolism were not affected by treatment, except acetate concentration, which was reduced with No-Thr. Mammary uptake of glucose, BHBA and lactate did not change with treatments, but acetate uptake increased with No-Trp infusion, in line with the numerical increase in fat yield. The reason why a Trp deficiency was driving an increased milk fat yield was not elucidated.

3.2 Future Directions

To improve the efficiency in milk protein synthesis beyond the current norm of 25-30%, we have to focus on the regulatory events of milk synthesis that occur in the MG. By identifying the factors that affect the maximum potential of milk secretion in the MG we can further improve N utilization in milk synthesis. Broad research areas that should be examined include: enzymatic reactions, AA limitations, AA transport and protein-energy interactions.

1. Enzymatic reactions: milk synthetic metabolic processes require enzymatic catalysis. We should consider molecular biological manipulations to activate those enzymatic reactions, because enzymatic reactions may be rate limiting in milk synthesis.
2. Amino acid deficiency will affect milk protein synthesis not only by reducing the supply of building blocks for milk protein synthesis, but also by reducing the rate of catalytic reactions through the potential reduction in enzyme synthesis. The EAA have a significant effect on different signalling pathways at the cellular level. It is necessary to examine whether the effects of AA deficiency occur due to substrate limitation, reduction of stimulation of signalling pathways, or both.

3. Amino acid transport into the mammary epithelial cells may limit milk protein synthesis; therefore, we have to focus on mammary AA transport mechanisms when presented with a variable EAA supply.
4. Milk synthesis is an energy-consuming event; therefore, future studies should simultaneously examine energy metabolism and protein metabolism. The level of ATP supply may be a major rate-limiting factor in milk protein synthesis. Factors that affect energy metabolism may also affect protein metabolism. For example, energy yielding metabolites can act as stimulatory or inhibitory molecules through cellular signalling pathways. Identifying cellular reactions that directly or indirectly control the rate of milk synthesis in the mammary epithelial cell will be very helpful in identifying limitations in milk synthesis.

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