Essential Amino Acid Regulation of Cell Signaling and Casein Synthesis in Mammary Tissue

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ABSTRACT

Specific AA have been demonstrated to activate signaling pathways that regulate translation initiation and to stimulate protein synthesis in mammary tissue. The objectives of this research were to determine the response to Ile, Leu, Met, and Thr in cellular signaling and α-S1 casein fractional synthesis rates (CFSR). An experiment was developed as a composite design. The experiment was replicated in tissue corresponding to 5 cows. Mammary tissue slices (0.12 ± 0.02 g) from lactating dairy cows were incubated 4 h in treatment media enriched with $^{2}H_5$ Phe. Following incubation, slices were homogenized in lysis buffer and caseins were precipitated by acidification to pH 4.6. An aliquot of the pellet was trypsinized and $^{2}H_5$ *Phe* enrichment in the 34-NLLRFFVAPFPE-45 peptide of α-S1 casein was measured by MALDI TOF-MS and used to determine CFSR (%/h). Western immunoblotting was performed to identify total and site-specific phosphorylated mammalian target of rapamycin (mTOR, Ser2448), eukaryotic elongation factor (eEF) 2 (Thr56), ribosomal protein (rp) S6 (Ser235/236), and eukaryotic initiation factor (eIF) 2α (Ser51). Addition of Ile, Leu, Met, or Thr had no effect on eIF2α phosphorylation. Isoleucine positively affected mTOR, and rpS6, and negatively affected eEF2 phosphorylation. Leu had a similar effect on eEF2, but not on mTOR or rpS6, and these two AA inhibited each other. Thr negatively interacted with Ile on mTOR and rpS6, and with Leu on eEF2. Increasing concentrations of Ile, Leu, Met, and Thr caused curvilinear increases in CFSR. The maximum response to Ile, Leu,

Met, and Thr was at 71, 49, 60, and 65% of DMEM concentrations, respectively. All maximums were above plasma AA concentrations observed in lactating cows fed to meet NRC requirements. The CFSR estimated at those maximums were similar between AA (3.6 ± 0.6 %/h). Individual AA effects on CFSR did not correlate with mTOR signaling. Independent CFSR responses to individual essential AA observed in this study contradict the single-limiting AA theory assumed in current requirement systems. The saturable responses of CFSR to these 4 AA also demonstrate the deficiencies of a fixed post-absorptive AA efficiency approach for determining AA requirements for milk protein synthesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: Introduction

Feeding the growing world population will be a challenging task because of the limited nature of resources. Management strategies as well as scientific contributions that increase food production are permanent targets. However, production and efficiency should not be improved at the expense of the environment or the gains will not be sustainable.

The dairy industry has experienced remarkable improvements in productivity over the past 50 years. This has resulted from genetic improvements in production traits, nutrient management, and lately animal welfare. Dairy operations in the US have evolved, increasing in size and individual cow milk production. The intensification of production systems has benefits for efficient management of resources, but also has disadvantages because negative impacts are magnified. Waste products released to the environment are among those problems that have increased and that require special attention. Among the dairy industry pollutants, N is of special concern because of its impact on the environment. Nitrogen export to the environment can result in eutrophication of aquatic ecosystems and coastal hypoxia; increased atmospheric particles, decreased stratospheric ozone concentrations, and greenhouse warming; increased acidity of precipitation, soil and surface water; and denigration of drinking water contributing to methemoglobinemia in infants (Wolfe and Patz, 2002).

Under current feeding practices (Caraviello et al., 2006), only one quarter of dietary N is incorporated into milk protein (Hristov et al., 2004). Considering the size of the US dairy industry, this means that 1.3 million metric tons of dietary N are excreted to the environment every year (305 d lactation). The two questions that these numbers elicit are: how much can N excretion be reduced, and how can it be achieved? About 60% of N losses happen post absorption (Hanigan et al., 2004). In swine, when AA supply matches demand, post-absorptive

efficiencies of 85% have been reported (Baker, 1996). Dairy cattle post-absorptive N efficiency for milk protein (MPE) averaged 44%, when a wide range of dietary CP levels was analyzed (Hanigan et al., 1998, Hristov et al., 2004, Lapierre et al., 2010). However, when cows are fed at 18% CP, that efficiency is reduced to approximately 37% (Whitelaw et al., 1986). Therefore, if dietary CP is reduced and specific AA are supplemented, MPE should at least reach 50%, which would reduce N excretion by 30%.

Post-absorptive N losses are mainly a consequence of splanchnic catabolism of AA. However, splanchnic affinity for AA (MacRae et al., 1997) is low compared to the mammary glands (Hanigan et al., 1992), so most of the AA absorbed in the small intestine reach peripheral circulation. Mammary use of AA depends on relative blood flow and mammary cell extraction. However, as extraction is less than 100%, AA not removed by the glands return to peripheral circulation (Hanigan et al., 1998). Splanchnic blood flow is between 3 and 4 fold greater than mammary blood flow (Delgado-Elorduy et al., 2002, Raggio et al., 2004), so large proportions of AA returned from peripheral tissues are pumped through the splanchnic tissues and subjected to removal and catalysis. Therefore, mammary extraction of AA were increased, recycling to splanchnic tissues would be reduced, and MPE would be increased. Amino acid extraction is not saturated over the normal range of arterial concentrations (Hanigan et al., 1992). Bequette et. al, (1996, 2000) demonstrated that extraction of individual AA can increase or decrease to meet demands. Hence, mammary extraction efficiency could be increased by reducing supply of AA while holding demand for milk protein constant, by increasing demand while holding supply constant, or a combination of the two.

During lactation, milk protein synthesis is regulated primarily at translation rather than at transcription (Lemay et al., 2007). Intracellular signaling pathways, including the mammalian

target of rapamycin (mTOR) and integrated stress response pathways, respond to nutrient and hormonal signals to regulate rates of mRNA translation. Specific essential AA have been found to activate the mTOR pathway and stimulate protein synthesis (Mahoney et al., 2009). In mammary tissue, branched chain AA have the most effect on cellular signaling and protein synthesis (Appuhamy et al., 2012, Haque et al., 2013, Moshel et al., 2006). These findings support the use of a feeding strategy similar to that used in swine, where dietary CP is reduced, and individual essential AA that cause the most response in protein synthesis are supplemented. Following that strategy should cause mammary extraction efficiency of AA to increase, and recycling to splanchnic tissue and N excretion to decline. However, the responses in milk protein yield to the AA that have shown an effect on cellular signaling and protein synthesis have not been quantified. Therefore the objectives of this research project were to quantify the responses in cellular signaling and milk protein synthesis to Ile, Leu, Met, and Thr, and to explore potential interactions between those AA for consistency with the single-limiting AA theory assumed by most nutrient requirement systems.

REFERENCES

Appuhamy, J. A. D. R. N., N. A. Knoebel, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2012. Isoleucine and leucine independently regulate mtor signaling and protein synthesis in mac-t cells and bovine mammary tissue slices. J. Nutr. 142(3):484-491.

Baker, D. H. 1996. Advances in amino acid nutrition and metabolism of swine and poultry. Pages 41-52 in Nutrient managment of food animals to enhance and protect the environment. E. T. Kornegay, ed. CRC Lewis, Boca Raton, FL.

Bequette, B. J., F. R. C. Backwell, J. C. MacRae, G. E. Lobley, L. A. Crompton, J. A. Metcalf, and J. D. Sutton. 1996. Effect of intravenous amino acid infusion on leucine oxidation across the mammary gland of the lactating goat. J. Dairy Sci. 79(12):2217-2224.

Bequette, B. J., M. D. Hanigan, A. G. Calder, C. K. Reynolds, G. E. Lobley, and J. C. MacRae. 2000. Amino acid exchange by the mammary gland of lactating goats when histidine limits milk production. J. Dairy Sci. 83(4):765-775.

Caraviello, D. Z., K. A. Weigel, P. M. Fricke, M. C. Wiltbank, M. J. Florent, N. B. Cook, K. V. Nordlund, N. R. Zwald, and C. L. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large us commercial farms. J. Dairy Sci. 89(12):4723-4735.

Delgado-Elorduy, A., C. B. Theurer, J. T. Huber, A. Alio, O. Lozano, M. Sadik, P. Cuneo, H. D. De Young, I. J. Simas, J. E. P. Santos, L. Nussio, C. Nussio, K. E. Webb Jr, and H. Tagari. 2002. Splanchnic and mammary nitrogen metabolism by dairy cows fed dry-rolled or steam-flaked sorghum grain. J. Dairy Sci. 85(1):148-159.

Hanigan, M. D., C. C. Calvert, E. J. DePeters, B. L. Reis, and R. L. Baldwin. 1992. Kinetics of amino acid extraction by lactating mammary glands in control and sometribove-treated holstein cows. J. Dairy Sci. 75(1):161-173.

Hanigan, M. D., J. P. Cant, D. C. Weakley, and J. L. Beckett. 1998. An evaluation of postabsorptive protein and amino acid metabolism in the lactating dairy cow. J. Dairy Sci. 81(12):3385-3401.

Hanigan, M. D., C. K. Reynolds, D. J. Humphries, B. Lupoli, and J. D. Sutton. 2004. A model of net amino acid absorption and utilization by the portal-drained viscera of the lactating dairy cow. J. Dairy Sci. 87(12):4247-4268.

Haque, M. N., H. Rulquin, and S. Lemosquet. 2013. Milk protein responses in dairy cows to changes in postruminal supplies of arginine, isoleucine, and valine. J. Dairy Sci. 96(1):420-430.

Hristov, A. N., W. J. Price, and B. Shafii. 2004. A meta-analysis examining the relationship among dietary factors, dry matter intake, and milk and milk protein yield in dairy cows. J. Dairy Sci. 87(7):2184-2196.

Lapierre, H., C. E. Galindo, S. Lemosquet, I. Ortigues-Marty, L. Doepel, and D. R. Ouellet. 2010. Protein supply, glucose kinetics and milk yield in dairy cows. Pages 275-286 in Energy and protein metabolism and nutrition. G. M. Crovetto, ed. Wageningen Academic Publishers, Wageningen.

Lemay, D., M. Neville, M. Rudolph, K. Pollard, and J. German. 2007. Gene regulatory networks in lactation: Identification of global principles using bioinformatics. BMC Syst. Biol. 1(1):56.

MacRae, J. C., L. A. Bruce, D. S. Brown, and A. G. Calder. 1997. Amino acid use by the gastrointestinal tract of sheep given lucerne forage. American Journal of Physiology - Gastrointestinal and Liver Physiology 273(6):G1200-G1207.

Mahoney, S. J., J. M. Dempsey, and J. Blenis. 2009. Chapter 2 cell signaling in protein synthesis: Ribosome biogenesis and translation initiation and elongation. Pages 53-107 in Progress in molecular biology and translational science. Vol. Volume 90. W. B. H. John, ed. Academic Press.

Moshel, Y., R. E. Rhoads, and I. Barash. 2006. Role of amino acids in translational mechanisms governing milk protein synthesis in murine and ruminant mammary epithelial cells. J. Cell. Biochem. 98(3):685-700.

Raggio, G., D. Pacheco, R. Berthiaume, G. E. Lobley, D. Pellerin, G. Allard, P. Dubreuil, and H. Lapierre. 2004. Effect of level of metabolizable protein on splanchnic flux of amino acids in lactating dairy cows. J. Dairy Sci. 87(10):3461-3472.

Whitelaw, F. G., J. S. Milne, E. R. Ørskov, and J. S. Smith. 1986. The nitrogen and energy metabolism of lactating cows given abomasal infusions of casein. Br. J. Nutr. 55(03):537-556.

Wolfe, A. H. and J. A. Patz. 2002. Reactive nitrogen and human health:Acute and long-term implications. Ambio 31(2):120-125.

CHAPTER 2: Current representation and future trends in modeling amino acid metabolic efficiency in the lactating dairy cow

ABSTRACT

In current dairy production systems only 25% of dietary N is captured in product, with the remaining being excreted in urine and feces. About two thirds of total N losses occur post-absorption. Splanchnic tissues extract a fixed proportion of splanchnic AA supply, and part of the extracted AA are converted into urea. Splanchnic affinity for individual AA is variable, but for most of the essential AA it is lower than mammary glands' affinity. Hence, most of absorbed AA reach peripheral circulation. The mammary glands receive nutrient and hormonal signals. Recent studies have demonstrated that AA, energy substrates, and hormones activate signaling pathways that in turn regulate local blood flow, individual AA extraction, and rate of mRNA translation. Therefore, extraction and utilization efficiencies of individual AA may vary to match nutrient supply with tissue demands. Excess AA are returned to general circulation, and due to splanchnic blood flow (three and four times mammary blood flow), a large proportion of these AA reach splanchnic tissues. Because of this splanchnic recycling and high splanchnic blood flow, AA catabolism by splanchnic tissues is often twice the ratio of incorporation into milk protein. Furthermore, mammary extraction efficiency decreases as supply increases. Therefore, the proportion returned to general circulation is not constant and thus the fractional mammary use of absorbed AA is not constant either. Dairy cattle nutrient requirement systems consider AA requirements in aggregate, and assume AA incorporation into product with a fixed efficiency. Particularly, the Dairy

NRC includes individual requirements for Lys and Met, but only as a proportion of the metabolizable protein supply, so the same efficiency is applied to all the AA (67%). By doing so, requirement systems limit the scope of diet manipulation to achieve improved N efficiency. Therefore, this review focuses on understanding nutrient dynamics in mammary and splanchnic tissues that would lead to improved requirement prediction equations. Inclusion of variable individual AA efficiencies deriving from splanchnic and mammary responses to nutrient and hormonal signals should help to reduce dietary protein levels. Supplementing low CP with individual essential AA should increase gross N efficiency to at least 30%, reducing N excretion by 260 mmt annually.
Key words: nitrogen requirement, amino acid, mammary gland, protein synthesis

INTRODUCTION

Agriculture in general and the dairy industry in particular is under increasing pressure from federal and local governments and from public opinion to reduce its environmental footprint. Among the dairy industry pollutants, N excretion is of major concern because of its impact on air and water quality, ecosystem biodiversity, and human health. Nitrogen export to the environment can result in eutrophication of aquatic ecosystems and coastal hypoxia; increased atmospheric particles, decreased stratospheric ozone concentration, and greenhouse gas concentration; increased acidity of precipitation, soil and surface water; and denigration of drinking water contributing to methemoglobinemia in infants (Wolfe and Patz, 2002). In addition to its environmental impact, protein, which is the source of the waste N, is an expensive dietary nutrient, representing approximately 42% of the cost of a lactating cow ration (St-Pierre, 2012). Improving N efficiency would positively impact the dairy industry economy and reduce demand for animal feed protein sources.

There are approximately 9 M dairy cattle in the US (Livestock, Dairy, and Poultry Outlook: August 2012, LDPM-218, Dairy Economic Research Service, USDA). In a survey carried out on 103 large dairies across the country (613 ± 46 cow and 34.5 ± 0.3 kg of milk sold per cow per day), nutritionists reported feeding diets which averaged 17.8 ± 0.1% CP (Caraviello et al., 2006). A meta-analysis using 846 experimental diets with similar average CP content reported a mean dietary protein to milk protein conversion of 24.6% (Hristov et al., 2004b). Thus, over a 10-month lactation, assuming the same dietary conditions (22.1 kg/d DMI and 17.8% CP) reported by Hristov et al. (2004b), 1.3 million metric tons (mmt) of dietary N would have been excreted.

To reduce the environmental impact of the dairy industry, it is necessary to know where the N is lost, the potential N efficiency that a dairy cow can reach, and what must be changed to get there. Dietary N that is not incorporated into product is excreted in feces or urine. Fecal N includes undigested dietary and microbial protein, and endogenous N losses. The latter represents between 18 and 31 % of the fecal N, and includes undigested secreted proteins, sloughed cells from the gut mucosa and urea recycled to the hindgut, which are not particularly responsive to the diet (Lapierre et al., 2008, Røjen et al., 2012). Duodenal digestibility varies on the fraction (microbial or dietary protein) and is reasonably repeatable for a given feedstuff. Rumen undegradable proteins tend to have lower digestibility (Erasmus et al., 1994) than microbial protein (Storm et al., 1983). Therefore, if most of the dietary protein provided is ruminally degradable and only high quality ruminally protected supplements are provided, fecal N excretion could be reduced.

Urinary N excretion is the most significant loss of N, and more important, it is possible to alter by diet manipulation (Lapierre and Lobley, 2001, Lobley et al., 2000a, Reynolds and Kristensen, 2008). Most of the urinary N is excreted as urea produced in the liver from two sources: ruminal ammonia and post-absorptive AA catabolism (Lobley et al., 2000b). The relative contribution of these two sources and the total amount of urea produced in the liver depend on how well N supply matches ruminal and post-absorptive requirements. When degradable protein is supplemented in excess extra N is absorbed as ammonia from the rumen and excreted as urea (Hristov et al., 2004a), but post-absorptive N efficiency does not change (Cyriac et al., 2008). On the other hand, rumen undegradable protein supplementation increases duodenal absorption of AA, and if

supplemented in excess, liver catalysis increases urea production. Therefore, by matching metabolizable protein (MP) supply with maintenance and lactation requirements, MP efficiency for milk protein (MPE) should increase, and urea production and urinary excretion should decrease.

Swine and poultry nutritionists formulate rations with reduced protein levels and supplement with specific AA to achieve dietary N efficiencies of 40% or greater. Baker (1996) demonstrated that post-absorptive use of N in pigs can be up to 85% when supply of AA matches tissue needs. Whether dairy cows can reach those efficiencies for milk protein synthesis is a question that we cannot answer with the current level of understanding of AA metabolism. However, reducing dietary CP to 14% or lower and supplementing with individual EAA should increase gross N efficiency to 30% and reduce N losses by 280 mmt/year. This, in turn, would reduce environmental impact and feeding costs, and free up to 2.2 M hectares of land previously used for soybean production (assuming 40 bushels/ac; http://www.nass.usda.gov/). Nutrient requirements need to be precisely defined to meet that objective. However, current requirement systems for dairy cattle are not adequate to adopt the nutrition strategy followed by swine and poultry nutritionists.

National Research Council (1989, 2001) equations are used in whole or in part by most ration balancing software packages in the US. Thus, the NRC model is used to determine protein supply in most US dairy diets. The NRC (2001) protein system predicts relatively accurate fluxes of AA to the small intestine. Absorbed AA are aggregated in the MP fraction, and proportional requirements for only Lys and Met are established. The MP is first used to meet maintenance requirements, which are functions

of body weight (endogenous urinary and scurf proteins) and DMI (metabolic fecal protein). The remaining is used for milk protein synthesis (when the cow is not pregnant or growing) with 67% efficiency until lactation requirements are met, and 0% efficiency following.

The NRC (2001) MPE is significantly higher than those reported in the literature (Hanigan et al., 1998, Hristov et al., 2004b, Lapierre et al., 2010). In an evaluation of that model (Figure 2.1), 82% of the studies had milk yields lower than the predicted MP allowable milk (NRC, 2001). The slope of residuals regressed on predicted was -0.34. Therefore, the actual MPE should be set 0.44 [0.67*(1-0.34)]. A quadratic term can be fitted ti the residuals also, suggesting that MPE decreases as MP supply increases. The authors identified energy and individual AA as the most likely nutrients to be limiting milk production (NRC, 2001). The reason for adopting higher MPE than those observed empirically may have to do with the compartmental nature and the maintenance requirements representation of the model. It assumes zero response in milk protein to MP until maintenance requirements are met. If this were not the case then it would under-predict milk protein yield (MPY) at maintenance. Also, maintenance requirements are described mainly as a function of body weight, with no variation due to level of production. If maintenance requirements change with production or supply of AA, then this approach increases the error at low MP levels. To fit those observations the slope has to be increased, over-predicting the response at MP levels close to lactation requirements, when responses in milk protein start to decrease (Hanigan et al., 1998). The later is more harmful than under-predictions at maintenance because most diets are formulated in that range of MP. Adjusting maintenance requirements and reducing the assumed MPE may

help to reduce MP content in dairy diets marginally, and hence reduce N excretion in urine. However, that does not increase the actual efficiency closer to values observed in swine and poultry.

Since the publication of the last revised edition of NRC (2001), a significant evidence has been published that highlights the deficiencies of a fixed MPE (Doepel et al., 2004). Studies indicate that splanchnic (Hanigan et al., 2004b, MacRae et al., 1997) and mammary tissues (Doepel et al., 2004, Hanigan et al., 2002) use individual AA at different rates. This can be partially explained by the nutrient and hormonal effects on signaling pathways that regulate local blood flow, AA membrane transport and mRNA translation. By treating AA in aggregate, MP requirements are set to supply enough of the AA with lower efficiencies for milk protein, while AA with higher efficiencies are supplied in excess. Finally, studies from the cellular (Appuhamy et al., 2011, Appuhamy et al., 2012) to the animal level (Haque et al., 2013, Rius et al., 2010, Toerien et al., 2010) indicate that individual AA and energy stimulate protein synthesis additively, contradicting the single-limiting AA theory assumed by most nutrient requirement systems. Therefore, the focus of this review is on AA metabolism studies, especially in the mammary glands (MG) that delineate modeling strategies to increase N post-absorptive efficiency.

POST-ABSORPTIVE N UTILIZATION

Essential AA cannot be synthesized in the body or cannot be synthesized at a rate that meets cellular requirements; so, they need to be provided by the diet. Determining individual AA requirements is a challenging task, especially in ruminants, and studies that contribute to that knowledge are still required. As a result, most nutrient requirement

systems for dairy cattle predict post-absorptive AA requirements in aggregate (MP). This implies that all AA are incorporated into product with the same efficiency. Particularly, the NRC (2001) includes Lys and Met requirements that are proportional to MP supply.

As previously mentioned, MP is first used to meet maintenance requirements and then incorporated into milk protein with 67% efficiency until lactation requirements are met, and 0% efficiency thereafter. However, 67% MPE is not observed empirically at wide ranges of CP supplementation. A meta-analysis across 846 experimental diets with CP ranging from 10.3 to 28.1%, reported an average MPE of 43.6% (Hristov et al., 2004b), similar to that estimated from the NRC evaluation. Specifically, increasing CP content from 13.5 to 19.4% DM produced no effect on MPY and rather resulted in a decrease in MPE from 52 to 43% (Olmos Colmenero and Broderick, 2006). Similarly, Holstein cows on an alfalfa silage-corn based diet supplemented with solvent extracted soybean meal or animal-marine blend had MPE ranging from 46 for low protein diets to 36% for high protein diets (Ipharraguerre et al., 2005). Looking at a wider range of dietary CP supplementation (11.3 to 20.1% DM), a quadratic response in MPY was observed, indicating that N efficiency decreases as supply increases (Metcalf et al., 1996b). In a very conclusive study, Whitelaw et al. (1986) observed that an abomasal infusion of 200 g/d of casein to cows fed 13.6% CP diet and producing 13 kg/d of milk, increased milk production by 2.3 kg with a marginal MPE of 41.5%. Infusion of an additional 200 g of casein/d increased milk production by another 1.2 kg/d and the marginal MPE decreased to 22.6%. Infusion of an additional 200 g of casein/d increased milk production by 0.7 kg and the marginal MPE was only 15.4 %. Since the cows responded to all the supplementations, they had not met lactation requirements, but the MPE was significantly

lower than the 67% assumed by NRC and clearly decreased as supply increased. Therefore, the assumption of a fixed MPE is not supported.

The reasons for a declining response to MP supply likely reflect the saturable nature of enzymatic processes, as well as the nature of the nutrient supply to the splanchnic tissues. Hanigan et al. (1998) pointed out that AA efficiency in a reaction is always 100%, which is not generally the case with energy efficiencies. If all the alternative AA use pathways had fixed efficiencies, MPE could be determined by the ratio of the milk protein synthesis affinity constant and the sum of affinity constants of the alternative pathways. Since most reactions are saturable, the function is mathematically more complicated, but the concept remains the same (Hanigan et al., 1998). From that concept, mammary AA uptake is negatively affected by splanchnic blood flow due to the negative impact of splanchnic use on overall AA supply, i.e. increased blood flow to the splanchnic tissues causes greater clearance and thus reduces systemic concentrations and AA supply to mammary tissue.

Figure 2.2 presents N fluxes (g/d) in lactating cows fed a low (15%) protein diet (Hanigan et al., 2004b). Post-absorptive N efficiency for milk protein was only 34%. Mammary first pass extraction (22%) of AA is greater than that of the PDV (4%) and liver (9%; Hanigan et al., 2004b, Raggio et al., 2004). As first pass mammary extraction is not 100%, those AA not used by the udder, and other peripheral tissues, are further recycled to the liver (Hanigan et al., 1998). Because splanchnic blood flow is between 3 and 4 times that of the MG (Delgado-Elorduy et al., 2002, Hanigan et al., 2004b, Raggio et al., 2004). Amino acid supply to splanchnic tissues is significantly higher than to the MG, and catabolism by the former can, and usually does, surpass incorporation into milk

protein (181 vs 98 g N/d respectively). Despite the predomination of systemic supply on splanchnic use of AA (MacRae et al., 1997), absorption and splanchnic catabolism of AA are positively related, which almost entirely reflects the impact of absorption on systemic concentrations. Thus, if mammary extraction could be increased with no change in rates of absorption, then recycling from peripheral tissues and splanchnic use of AA would be reduced with no loss in production.

Metabolizable Protein or Individual AA Requirements

The next logical question is if variable partial efficiencies can be homogenously applied to all AA, or if differences on individual AA utilization are great enough to require the use of individual efficiencies also. An aggregated AA requirement would be valid if the EAA profile of the RUP plus microbial protein were not variable, and if the absorbed mix of EAA was not affected by tissue metabolism and generally was well matched to tissue needs. However, if these conditions were not met, a MP-based model would not be able to capture milk protein responses to changes in the AA profile. It would also inappropriately predict responses to MP supply when individual AA deficiencies prevent them.

Clark et al. (1992) reported large variation in AA composition of ruminal bacteria. However, part of that variation was attributed to laboratory technique and site and time of sampling, and no specific dietary factor was identified as responsible for changes in microbial AA composition. Similarly, Boguhn et al. (2006) studied the effect of 16 total mixed rations on microbial AA composition, and despite variation in microbial AA composition, those difference could not be attributed to dietary nutrients. However,

forage fiber content marginally affected microbial AA composition *in vitro* (Rodríguez-Prado et al., 2004).

Conversely, dietary proteins vary in AA composition and digestibility (Piepenbrink and Schingoethe, 1998), and can affect the blood AA pool. It is well documented that corn-based diets do not supply enough Lys to lactating cows (Schwab et al., 1992a, Schwab et al., 1992b, Wu et al., 1997). Soybean-supplemented cows have been observed to respond to Met (Armentano et al., 1997, Noftsger and St-Pierre, 2003). Also, for grass or cereal-fed cows, responses to His have been reported (Korhonen et al., 2000, Vanhatalo et al., 1999). In a conclusive study, Haque et al. (2012) demonstrated that correcting the intestinal AA profile without changing protein supply modified blood AA concentrations and improved milk production, MPY, and MPE. The latter improved by 8% reaching, for one of the treatments, 50% MPE. Therefore, observed variations in rumen undegradable and possibly in microbial protein AA composition can affect AA absorption, and this is reflected in milk and milk protein production. An MP based model is not able to predict the response observed by Haque et al (2012) because it does not consider individual AA requirements and it does not predict possible changes in MPE.

However, even if the ideal AA profile is provided to the duodenum, AA can still be used by splanchnic tissues for constitutive or export protein synthesis, or catabolized and the N converted to urea (Raggio et al., 2007). Such use is driven primarily by the return of AA not used by peripheral tissues to general circulation. In fact, most of the AA used by PDV come from arterial circulation rather than intestinal absorption (MacRae et al., 1997). Lapierre (2005) reported large differences in liver extraction between EAA, and classified these in two main groups. Lys and BCAA are barely used by the liver,

meanwhile, Phe, Met and His are removed at high rate (Lapierre et al., 2005). Hence, splanchnic extraction of individual AA impacts MG supply and utilization, and an aggregated model cannot capture these variations.

Utilization of EAA by the Mammary Glands. Even if the ideal AA profile reaches the MG, the success of an aggregated model will depend on the glands' efficiency to extract individual AA. Mammary extraction of AA changes with arterial concentration (Bequette et al., 2000, Doepel et al., 2004), physiological (Schwab et al., 1992a) and hormonal status (Mackle et al., 2000), and tissue needs (Ipharraguerre and Clark, 2005). Mammary AA extraction is a function of arterial concentration and blood flow (Baumrucker, 1985). The parameters of that function depend on AA transporter affinity for individual AA. The mathematical representation of mammary AA extraction by Hanigan et al. (1998) demonstrates that supply is a function of AA absorption from the gut, tissue relative affinities, and blood flow. Therefore, tissue clearance rate is a better indicator of extraction than arterio-venous difference, because the latter fails to consider the effect of blood flow.

Several studies have reported local effects of nutrients on mammary blood flow. In lactating rats, 18 h feed deprivation reduced mammary blood flow to half (Stewart et al., 2009). Refed rats recovered mammary blood flow to initial levels within 60 min. When the supply of only one AA is reduced, the MG locally increased local blood flow to provide more of the deficient AA. Bequette et al. (1996, 2000) observed 17 and 36% increases in mammary blood flow when EAA mixtures depleted of Leu or His were infused to lactating goats, respectively. In dairy cows fed a 16% CP diet, arterial infusion of 30 g AA mix with or without His did not affect mammary blood flow (Cant et al.,

2001). However, His arterial concentration in the His depleted infusion treatment did not differ from the control (saline infusion), and MPY was similar to both the control and the complete AA infusion, suggesting that His was sufficient in the diet. Guinard and Rulquin (1995) used an addition approach to investigate the effect of Met on mammary blood flow and observed that provision of 16 g of Met to dairy cows producing 24 kg/d of milk significantly reduced blood flow, but that effect disappeared when the Met dose was doubled. Increasing levels of Lys up to 63g/d increased arterial Lys but had no effect on blood flow (Guinard and Rulquin, 1994). Conversely, abomasal infusion of all the EAA (359 g/d) reduced (5%) blood flow to the MG, whereas a non-EAA infusion (356 g/d) tended to increase (12%) blood flow (Doepel and Lapierre, 2010). Increasing the supply of RUP had no effect on mammary blood flow (Metcalf et al., 1996b), which agrees with the response in the combined EAA plus non-EAA treatment of Doepel and Lapierre (2010). Provision of glucose increased mammary blood flow linearly, affecting the uptake of AA (Rulquin et al., 2004). Rius (2010) observed an increase in mammary blood flow and nitric oxide synthase phosphorylation (indicating enzyme activity) in response to abomasal infusion of starch. The mechanism behind nitric oxide synthase activation is unclear, but it is not likely a local effect of insulin because no effect on Akt phosphorylation was observed (Rius et al., 2010). From these observations, it appears the MG regulates local blood flow in response to nutrient deficiency or imbalance, affecting nutrient partitioning.

Blood flow has been described as a linear function of milk yield (Hanigan et al., 2002, Kronfeld et al., 1968). Guinard and Rulquin (1995) observed variation in the blood flow-milk output ratio with Met infusion. Cant and McBride (1995) represented mammary

blood flow as a negative function of intracellular ATP/ADP ratio. Based on their model, increasing blood nutrient concentrations (AA, acetate, and glucose) was predicted to reduced blood flow due to an increase in the ATP/ADP ratio. Conversely, reducing nutrients concentrations increased blood flow and clearance per unit of time of AA, fatty acids and glucose, but not acetate by the MG. That effect has been experimentally observed for infusions of EAA (Doepel and Lapierre, 2010), but not for non-EAA, total AA (Metcalf et al., 1996a) or glucose (Rulquin et al., 2004). A more comprehensive representation of local blood flow regulation in the MG has not been published. Further research is needed to understand the mechanisms controlling blood flow regulation in the MG and other tissues if we are to achieve a better description of variable partial efficiencies in a post-absorptive N metabolism. We anticipate that local blood flow regulation is a non-linear function of at least several independent nutrients as happens with milk protein synthesis (chapter 4). However, data availability does not currently allow parameterization of such a representation.

Mammary extraction efficiency (A-V difference as a percent of arterial concentration) of AA in lactating Holstein multiparous cows ranged from 5% for Asp to 69% for Phe (Hanigan et al., 1992). The average efficiency for EAA was 43%, and for non-EAA was 30% (Hanigan et al., 1992). A mass action representation (fixed clearance rate) for individual AA explained much of the variation in venous concentration for all AA except glutamate, and there was no apparent bias in predictions (Hanigan et al., 2002). However, lactating cows fed below MP requirements (13.9% CP diet) had an EAA extraction efficiency of 42%, which decreased to 32% when the cows were infused in the abomasum with 359 g/d of an EAA mix (Doepel and Lapierre, 2010). Most EAA were

affected except for Phe and Trp. Bequette et al. (2000) observed a 440% increase (17 to 74%) in mammary extraction efficiency for His when arterial His concentration was reduced by 90% in lactating goats fed 10.5% dietary CP and infused with a mixture of EAA devoid of His. That effect was not observed in lactating cows fed 16% CP and arterially infused with 300 g/d of AA mix with or without His (Cant et al., 2001), but it is likely that His was sufficient because MPY was similar between the two infusions. Abomasal infusion of casein plus branched-chain AA (BCAA; 500 and 88 g/d, respectively) to lactating cows fed above MP requirements reduced extraction efficiency of Val (Mackle et al., 2000). In the same study, insulin (hyperinsulinemic-euglycemic clamp) increased extraction efficiency for BCAA plus Lys. Therefore, although a fixed clearance rate explained venous concentrations of AA in cows with small to moderate perturbations of supply, it is clear that it does not completely represent the biology of the system and fails when challenged with data from animals where large perturbations in they system have been imposed, such as the case for the work of Bequette et al. (2000) and Doepel and Lapierre (2010). Clearly the transport system is regulated to at least partially match clearance rates with intracellular needs (Hanigan et al., 2000).

Mammary AA extraction potential depends on the number of epithelial cells and the transport activity of those cells. The number of cells is not likely to explain short-term changes in extraction rate observed upon abomasal or arterial AA infusions. Transport of AA across the epithelial cell membrane is an active process that depends on the number of transporters located in the membrane, and transport kinetics. Short-term responses in transport activity are related to relocation of AA transporters from intracellular compartments to the plasma membrane, whereas long-term adaptations are a result of

gene expression changes for those transporters (Mackenzie and Erickson, 2004). Amino acid transporters in mammary epithelial cells function below saturation, so changes in transport rate in response to changes in AA concentrations at the cell surface (driven by plasma AA concentrations and blood flow) are expected purely based on kinetics (Baumrucker, 1985).

There are seven AA transport systems: four (ASC, A, L, Y^+) are known to be present in bovine mammary epithelial cells but evidence for another two (N and anionic) is inconclusive (Baumrucker, 1985). Tight regulation of AA transport system A has been observed in relation to intracellular AA concentrations and insulin signaling in other tissues (Mackenzie and Erickson, 2004). In mammary cells, insulin increased the expression of the Y^+ transporter gene (Menzies et al., 2009), and prolactin seemed to affect transport activity of systems A and L (Shennan et al., 1997). Figure 2.3 depicts gene expression regulation of proteins belonging to the cationic and neutral Na^+ dependent and independent transport systems (SNAT-2, CAT-1, LAT1, and EAAT). These genes have been shown to respond to intracellular AA concentrations through the integrated stress response (ISR) pathway (Kilberg et al., 2005). The same pathway has been shown to stimulate protein translation initiation in liver (Anthony et al., 2004) and mammary epithelial cells (Arriola Apelo et al., 2010). In human skeletal muscle, Leu supply stimulated Leu transport into the cell by up-regulating mRNA expression of the AA transport system L genes LAT-1, CD-98, SNAT-2, and PAT-1, and mRNA translation of LAT-1 and SNAT-2 (Drummond et al., 2010). In this study, activating transcription factor (ATF) 4 expression was increased despite no effect on eukaryotic initiation factor (eIF) 2α (Ser 51) phosphorylation. The authors suggested an effect of

mammalian target of rapamycin complex 1 (mTORC1) on AA transporter genes expression (Drummond et al., 2010). If true, this provides a linkage between cellular AA demand and supply. Such a mechanism would explain observed changes in clearance rates of individual AA as noted in studies reported above. It is also consistent with the observation that supplementation of a single EAA can stimulate milk protein synthesis (Hanigan et al., 2000). This would not be possible if blood flow or individual AA clearance rates were not variable, i.e. the other AA required to support increased production could not be obtained. However, more research is needed to understand and connect local mechanisms of regulation of AA extraction by the MG with milk protein responses. That knowledge would allow formulation of equations that could accurately predict variations observed when the supply of individual AA is affected. In spite of that, the studies reviewed clearly indicate the deficiencies of an AA aggregated model because the AA profile inside the mammary cells at the time of milk protein synthesis is highly variable. However, the applicability of an MP based model will depend, at the end, on whether the cells are responsive to changes in the AA profile.

Mammary Cell Demand and Nutrient Signaling

Coordination of mammary AA extraction with cellular demand helps prevent wasteful energy expenditure associated with AA transport, ribosome biogenesis and translation. Protein synthesis is tightly regulated at the transcriptional and translational level. Positive stimuli that increase protein synthesis rates have a negative effect on intracellular free AA concentrations and tRNA loading, which needs to be alleviated by increasing AA transport activity. Therefore, cellular coordination between AA demand and extraction seems indispensable.

Milk protein synthesis is regulated at transcription level by the lactogenic hormones prolactin and glucocorticoid (Doppler et al., 1989). However, after initiation of lactation milk protein genes reach a steady state and most of the regulation happens post-transcriptionally (Lemay et al., 2007). In agreement with these observations, the protein kinase B (Akt) pathway that mediates insulin effects on mRNA translation is up-regulated during lactation (Lemay et al., 2007).

Translation of milk protein mRNA is regulated via the activity of initiation and elongation factors. Nutrients and hormones activate signaling pathways that control the activity of these factors. The mTOR pathway is the translational regulation pathway most studied. The mTOR protein is a serine-threonine kinase that exists in two different complexes: mTORC1 and mTORC2. The former contains mTOR, Raptor, and mLST8 (Mahoney et al., 2009). The mTORC1 regulates the rate of protein translation and cell growth (Figure 2.4). The mTORC2 is an Akt kinase that contains, in addition to mTOR and mLST8, Rictor, S1N1, and PRR5/PRR5L (Dos et al., 2004, Gan et al., 2011). The mTORC1 phosphorylates eIF4E-binding protein 1 (4E-BP1) and ribosomal protein S6 (**rpS6**) kinase 1 (**S6K1**). When unphosphorylated, 4E-BP1 antagonizes eIF4G binding to eIF4E and inhibits translation (Mahoney et al., 2009). S6K1 phosphorylates rpS6 and eIF4B, which promote translation initiation. Another target of S6K1 is eukaryotic elongation factor (**eEF**) 2 kinase. This kinase is inhibited by phosphorylation, resulting in activation of eEF2 and promotion of translation elongation (Dunlop and Tee, 2009). In mammary tissue of lactating cows, phosphorylation of mTOR, 4EPB1, and eEF2 was positively correlated with synthesis rate of casein (Appuhamy et al., 2011).

Insulin and growth factors, including IGF-1 are indicators of positive energy status of the animal, and as such, they stimulate anabolic processes like protein synthesis in peripheral tissues (Davis et al., 2002). Insulin binding to its membrane receptor sequentially activates the Akt pathway (Bellacosa et al., 1998). In MAC-T cells, but not in mammary tissue slices, insulin stimulated phosphorylation of insulin receptor substrate 1 (**IRS1**), AKT, and downstream proteins (Appuhamy et al., 2011).

Local energy status is also sensed intracelularly by the AMP-activated protein kinase (**AMPK**), which detects increases in the AMP/ATP ratio (Hardie, 2004). Tuberous sclerosis complex (**TSC**) integrates AKT and AMPK signals (Inoki et al., 2002, Inoki et al., 2003b), and inactivates the small G-protein Ras homolog enriched in brain (**Rheb**; Inoki et al., 2003). Rheb enhances mTORC1 kinase activity by releasing FK506-binding protein (**FKBP**) 38 from the complex (Bai et al., 2007). Furthermore, AKT also induces proline-rich AKT substrate 40 kDa (**PRAS40**) binding to 14-3-3 protein, and promotes substrate binding to mTORC1 (Vander Haar et al., 2007, Wang et al., 2007). In contrast, activated AMPK phosphorylates Raptor, promoting the binding of 14-3-3 and PRAS40 proteins to mTORC1 (Gwinn et al., 2008). Therefore, mTORC1 integrates animal and cellular signals to ensure proper matching of cellular activity with energy availability.

The mTORC1 also receives inputs from EAA, what makes complete sense because these are the substrates for protein synthesis. Much progress has been made recently in understanding AA effects on mTORC1 activity. Essential AA, specifically Leu, activate Rag GTPase to recruit mTORC1 to the lysosomal membrane, where it interacts with Rheb (Sancak et al., 2010). Specifically, leucyl-tRNA-synthase in the presence of Leu or other AA functions as a GAP for Rag D to recruit mTORC1 to the lysosomal and activate

it (Han et al., 2012). Appuhammy et al. (2012) observed effects of Ile as well as Leu on mTOR phosphorylation and casein fractional synthesis rates (CFSR) in mammary tissue. In agreement, Moshel et al. (2006) reported that mTOR signaling in mammary epithelial cells was more sensitive to all AA than to Leu alone, and this effect was reflected in synthesis rates of ß-lactoglobulin. Recently, it was reported that a Val deficient diet limited milk protein synthesis in dairy cows (Haque et al., 2013). However, it is unknown whether the observed effects were due to Val as a substrate or via its cell signaling effects.

The ISR pathway is an mTOR-independent mechanism of translation regulation also regulated by essential AA. Four distinct kinases activate this pathway in response to different environmental threats. Particularly, general control non-derepressable (**GCN**) 2 senses AA depletion by binding with deacylated tRNA, and subsequent phosphorylation of eIF2α. Phosphorylation of eIF2α blocks the release of GDP upon hydrolysis from eIF2 and stops methionyl-tRNA recruitment to the 40 S ribosomal subunit (Figure 2.3). In MAC-T cells, His and BCAA supplementation reduced eIF2α phosphorylation (Arriola Apelo et al., 2010). However, in mammary tissue slices no effect of these AA on eIF2α phosphorylation was observed despite changes on milk protein synthesis rates (Appuhamy et al., 2012).

By coordinating EAA and energy supply with demand, mammary cells avoid the potentially costly mistake of synthesizing secretory components when inadequate substrate supply, or failing to secrete enough components when there is an excess of substrate. This cellular strategy opens the possibility for nutritional manipulation by reducing dietary protein supply and supplementing with specific AA that activate the

corresponding signaling pathways. This strategy would maximize rates of protein synthesis and minimize AA recycling to the liver thus improving AA efficiency and reducing N excretion.

Several efforts to model nutrient metabolism at tissue level have been undertaken (Hanigan et al., 2001, Hanigan et al., 2004a, Hanigan et al., 2004b). Incorporation of cellular signaling regulation into those models seems to be the next logical step to reach the final objective of increasing nutrient efficiency and reducing environmental impact of the dairy industry. Initial attempts developed a comprehensive model of mRNA translation, which included predictions of signaling protein activation by EAA and insulin, and protein synthesis rates (El-Haroun et al., 2010). Parameter values were derived from the literature, but the model has not been validated with independent data. Other efforts include mechanical models of translation that do not include nutritional regulation, so they lack applicability with respect to our objective (Basu and Chowdhury, 2007, Sharma and Chowdhury, 2011, Tuller et al., 2010).

There have been attempts also to represent individual signaling pathways. A model of the mTOR pathway activation by AA and insulin, and negative feedback on the insulin pathway was recently developed (Vinod and Venkatesh, 2009). The model predicted saturable responses in mTOR and S6K1 phosphorylation with respect to supply of AA, and additive effects of insulin. Interestingly, it also includes a negative feedback of AA on the insulin pathway and glucose transporter GLUT4 translocation to the cytoplasmic membrane. Similar to protein synthesis models presented above, this model requires specific inputs that limit its use in nutritional research. Appuhamy and Hanigan (2010), represented the mTOR pathway activation in a simplified representation with 3 proteins

(Akt, mTOR, and 4EBP1) and 6 pools (phosphorylated and unphosphorylated for each protein). Phosphorylation was driven by mass action as a function of insulin and EAA concentrations (Figure 2.5). Phosphorylation of downstream proteins (mTOR and 4EBP1) was estimated with minimum error (< 10%). As additional data are generated, this model could be incorporated into existing tissue models and evaluated for accuracy on prediction. Although, this level of complexity should not be required for incorporation into animal requirement models for production purposes. However, this molecular work helps to identify sources of variation in response variables and provides a quantitative understanding of the importance of these mechanisms with respect to deficiencies in existing systems. Through such an understanding, we can gain knowledge required to construct more aggregated and empirical representations of the key elements for use in our requirement systems.

NUTRIENT INTERACTIONS

The first limiting AA theory applied to animal nutrition is based on the Law of the Minimum which derives from the work by the German botanist Carl Sprengel in 1828. The original hypothesis stated that a nutrient can limit plant growth, and when limiting, growth will be proportional to supply. von Liebig (1863) subsequently restated the hypothesis in stronger terms indicating that if a nutrient was limiting for growth, responses to other nutrients could not occur. Mitchell and Block (1946) used von Liebig's extension of Sprengel's hypothesis to develop the concept of the order of limiting AA which is commonly described using the analogy of a water barrel with broken staves, where only the AA represented by the shortest stave can cause a response (Cant et al., 2003). Based on this concept, if an AA is limiting milk production, then,

only the addition of that AA to the diet will result in a positive milk yield response, e.g. the single-limiting AA theory. Several studies at the animal level have challenged this theory, while others agree with it (Cant et al., 2003). Hanigan et al. (2000) observed that the effects of individual AA on milk protein synthesis were better represented by multi-amino acid Michaelis-Menten model than the single-limiting AA approach.

Milk protein synthesis is a multi-step enzymatic process that happens in the cell at a rate determined by substrate availability and enzyme catalytic potential. Essential AA can limit milk protein synthesis by activating signaling proteins that modify enzyme catalytic potential, or by increasing tRNA loading and thus substrate availability. It was previously assumed that tRNA loading was not limiting for protein synthesis (reviewed by Cant et al., 2003). However, there have been observed responses in milk protein synthesis to EAA without activation of signaling pathways (Appuhamy et al., 2012). Elf et al. (2003) demonstrated that when an AA becomes limiting the charging level of some iso-acceptor tRNA can approach zero while others remain fully loaded. The rate of translation of the respective codons could thus differ greatly due to substrate availability. This study also showed that iso-acceptor sensitivity to AA deficiency and codon usage (frequency of appearance in genes expressed in the cell) were not necessarily correlated. The concept of differential iso-acceptor loading validates the multi-substrate application of the Michaelis-Menten equation for protein synthesis (Hanigan et al., 2002). It also agrees with early studies where addition of more than one AA (Clark et al., 1978) or groups of AA (Park et al., 1976) to mammary explants independently increased the rate of milk protein synthesis. At that time, the signaling pathways that regulate protein synthesis had not being described, so, it cannot be determined if the responses were due

to substrate or signaling effects of AA. However, recently, Appuhamy et al. (2012) observed no effect of Met and Thr on signaling proteins in MAC-T cells, and mammary tissue slices, but these AA did affect casein synthesis in mammary tissue. Below are presented the effects of Ile, Leu, Met and Thr on signaling pathways (chapter 3), and CFSR (chapter 4) in mammary tissue slices. Interestingly, AA stimulated signaling pathways and protein synthesis at different concentrations. Furthermore, the effect of individual AA on protein synthesis was additive (interactions were not significant) contradicting the single-limiting AA theory for protein synthesis in a mammary tissue model.

However, signaling pathways that regulate translation initiation and elongation have been shown to respond to specific AA. In mammary tissue slices, only Leu and Ile have been shown to activate the mTOR pathway. Activation of mTOR increases maximum translation rate without changing ribosomal affinity for tRNA. By increasing the maximum catalytic rate (V_{max}) without changing substrate affinity (k_m) at a given concentration of substrate, catalytic rate (mRNA translation) increases. Therefore, Leu or Ile could stimulate protein synthesis even when they are not considering first limiting according to the hypothesis. This is a violation of the single-limiting AA theory because the cell may respond to not only Leu and Ile, but perhaps also other AA that are decreasing translation rates due to low substrate availability. Moreover, mTOR can also be activated by hormonal or intracellular energy signals, thereby either causing an increase in activity in the absence of a change in the "limiting nutrient" or preventing an increase in activity when more of the "limiting nutrient" is provided.

Post-ruminal infusion of casein and glucose produced an additive response in MPY in lactating cows (Vanhatalo et al., 2003). This response can be partially explained by an increase in arterial concentrations of some EAA due to reduced hepatic use (Vanhatalo et al., 2003). However, Rius et al. (2010) determined that part of the response to increased post-ruminal glucose (as starch) supply was explained by changes in phosphorylation state of the mTOR pathway proteins (rpS6, and partially mTOR) in response to increased concentrations of blood glucose or insulin. In MAC-T cells, EAA and insulin additively activated the mTOR pathway (Appuhamy et al., 2011). Furthermore, in MAC-T cells and mammary tissue slices, individual EAA independently affected the phosphorylation state of mTOR (Appuhamy et al., 2012) . These changes were correlated, in mammary tissue, with changes in rate of casein synthesis.

Therefore, individual EAA independently stimulate rates of milk protein synthesis, while specific AA and energy supply activate signaling pathways that also stimulates it, contradicting the single nutrient theory. Thus, additive nutrient effects, in addition to variable partial efficiencies, should be included in nutrient requirement models to fairly represent individual responses to EAA and energy, and in this way reduce N requirements.

SPLANCHNIC USE OF AA

Free or peptide-bound AA are absorbed from the lumen of the small intestine (MacRae et al., 1997, Tagari et al., 2008). First pass extraction of AA by the PDV and liver is low compared to that of the MG (Hanigan et al., 2004b, MacRae et al., 1997). However, blood flow is about three times greater in the splanchnic tissue (Raggio et al.,

2004). As a result, on a daily basis splanchnic tissue uses about 50% of absorbed AA, representing the most significant post-absorptive N loss (Hanigan et al., 2004b).

Among the factors that affect splanchnic use of AA are intestinal absorption, arterial plasma concentrations, blood flow, and tissue demand. Blood flow in the portal vein responds to energy supply (Lomax and Baird, 1983) but not to protein supply (Hanigan et al., 2004b, Raggio et al., 2004). As the PDV is a net exporter of AA, increased blood flow would reduce portal and interstitial AA concentrations, and thus catabolism by the tissue bed (Hanigan et al., 2004b). Hence, the increases in blood flow mediated by energy supply would increase the release of AA to peripheral tissues.

Assuming gut growth could be considered quantitatively insignificant in a lactating animal, AA use by the PDV has three main components: synthesis of export proteins, replenishment of sloughed cells, and energy supply from AA carbon skeletons. Export protein synthesis is estimated to represent between 0 and 50% of total endogenous N losses, with the remaining being represented by sloughed cells (Lapierre et al., 2008). If energy supply does not affect endogenous N losses, variations observed in PDV utilization of AA in response to energy supply would correspond with changes in use of AA carbon skeletons. Therefore, greater blood flow caused by increased energy supply would reduce PDV catabolism of AA and use of their carbon skeletons as energy sources, increasing their release to the portal vein and peripheral circulation. If AA were limiting milk protein synthesis, energy would help to alleviate that limitation, and by supplying more AA stimulate protein synthesis rates in the MG. If in addition, more AA are supplied in the diet, the effects of energy and AA on milk protein synthesis would be additive at the animal level. The opposite is expected to happen when the supply of

dietary N increases. A high protein diet would increase AA absorption by the gut without affecting portal blood flow, causing higher interstitial concentrations and greater catabolism of AA by the PDV. Hence, dietary manipulation in several ways could affect PDV utilization of AA and supply to peripheral tissues.

Splanchnic tissue affinity for individual AA has not been shown to fluctuate with AA concentrations. Thus, AA catalysis rates are expected to be proportional to interstitial or intracellular AA concentrations (by mass action). Furthermore, peripheral circulation is the main supplier of AA to the PDV (Hanigan et al., 2004b). Hence, increased blood flow would increase influx and catabolism of the AA by the PDV. A mathematical representation of PDV extraction of AA as a linear function of supply has been successful in predicting portal concentrations of individual AA (Hanigan et al., 2004b). Prediction errors were below 10% of the observed concentration in the portal vein.

Portal drained viscera utilization of AA is part of the maintenance requirement. The effect of energy and protein on blood flow and splanchnic utilization of AA suggests that a proportion of this maintenance requirement varies with nutrient supply. This variation, in addition to variable efficiency in the mammary glands may explain most of the observed NRC (2001) over-prediction of MP requirement.

Similarly, blood flow across the liver decreased when total supply of dietary nutrients was decreased (Lomax and Baird, 1983). However, abomasal infusion of casein had no effect on it (Hanigan et al., 2004b). Amino acid metabolism by the liver (assuming no net growth) is related to export proteins and urea synthesis. The first one is not affected by supply of AA (Raggio et al., 2007). Urea production increases in response to ruminal

ammonia absorption, absorbed supply of AA from the gut, and return of unused AA from peripheral tissues.

Branched chain AA and Lys are removed by the liver at a minimal rate. Conversely, between 35 and 50% of Met, Phe and His supply is captured on a daily base (Lapierre et al., 2005). Experimentally, net hepatic uptake of Phe increased with supply in a high MP diet, offsetting the higher supply from portal circulation (Raggio et al., 2007). This resulted in similar releases from splanchnic tissue to peripheral circulation between a high and a low MP diet. Hanigan (2004a) represented liver uptake of AA as a function of supply. The derived clearance rates were several fold greater for His, Met and Phe than for Ile, Leu and Val. Extraction efficiency of Phe, His and Met by the liver were similar to those predicted for the MG (Hanigan et al., 2001). Given that the liver receives about four times more blood flow, this indicates that the affinity of the liver for those AA is significantly lower, and it is the recycling from peripheral tissues that accounts for much of the liver uptake of AA. From an evolutionary stand point, this is a logical strategy because the liver can sense peripheral AA status and respond accordingly. Nutritionally, it is an opportunity because it allows stimulation of protein synthesis in the MG with energy or signaling AA that are minimally removed by the liver (BCAA), increasing mammary uptake of other AA, and reducing liver uptake and catabolism.

CONCLUSIONS

Current understanding of AA metabolism is adequate to allow the improvement or development of nutrient requirement systems for dairy cattle that not only should reduce prediction errors, but also incorporate metabolic mechanisms, increase the robustness of the system as pillar for future contributions. To better represent the biology behind the

milk and milk protein synthesis processes, an improved system should include variable partial efficiencies that capture the saturable nature of enzymatic reactions as protein synthesis. Variable efficiencies are a function, in part, of local regulations of nutrient partitioning. Among those mechanisms, local regulation of blood flow has been demonstrated to intervene on the natural relation of tissue affinities that determine nutrient partitioning. Less significant but still encouraging have been the contributions to the understanding of AA transport regulation in mammary cells. It is still necessary to identify at a molecular level the systems that transport each of the AA, the overlap between them and the mechanisms of regulation. However, it is clear from infusion studies that they can respond to changes in AA supply and at least partially mitigate them. Interestingly, AA transport systems have been shown to coordinate with intracellular signaling pathways to match nutrient availability with tissue demand for protein synthesis. The progress obtained in the understanding of signaling pathways, their mechanisms of activation and their effect especially on translation regulation have been remarkable. Furthermore, understanding these pathways has contributed evidence at cellular level to the concept that individual AA and energy can independently stimulate protein synthesis, in agreement with observations at the animal level. Such responses are inconsistent with the single-limiting AA theory. Differential responses to individual AA limit the use of an aggregated representation of AA requirements. This approach fails to capture milk protein responses to corrections in dietary AA profile without changing total N supply. It also fails to capture variable utilization efficiencies between tissues, especially splanchnic, that modify dietary AA profiles. Requirements are inflated

because protein must be supplied at a level to meet the needs of those AA used with the least efficiency relative to supply.

Incorporation of these concepts into future requirement systems should allow formulation of rations at lower CP levels with selected supplementation with specific AA. This approach would increase post absorptive partial efficiencies and reduce N excretion to the environment, thus improving the dairy industry economy and public image.

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REFERENCES

Anthony, T. G., B. J. McDaniel, R. L. Byerley, B. C. McGrath, D. R. Cavener, M. A. McNurlan, and R. C. Wek. 2004. Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eif2 kinase gcn2. J. Biol. Chem. 279(35):36553-36561.

Appuhamy, J. A. D. R. N. and M. D. Hanigan. 2010. Modeling the effects of insulin and amino acids on the phosphorylation of mtor, akt, and 4ebp1 in mammary cells. Pages 225-232 in Modelling nutrient digestion and utilisation in farm animals. D. Sauvant, J. V. Milgen, P. Faverdin, and N. Friggens, ed. Wageningen Academic Publishers, Wageningen, The Netherlands.

Appuhamy, J. A. D. R. N., A. L. Bell, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2011. Essential amino acids regulate both initiation and elongation of mrna translation independent of insulin in mac-t cells and bovine mammary tissue slices. J. Nutr. 141(6):1209-1215.

Appuhamy, J. A. D. R. N., N. A. Knoebel, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2012. Isoleucine and leucine independently regulate mtor signaling and protein synthesis in mac-t cells and bovine mammary tissue slices. J. Nutr. 142(3):484-491.

Armentano, L. E., S. J. Bertics, and G. A. Ducharme. 1997. Response of lactating cows to methionine or methionine plus lysine added to high protein diets based on alfalfa and heated soybeans. J. Dairy Sci. 80(6):1194-1199.

Arriola Apelo, S. I., W. A. D. Nayananjalie, J. A. D. R. N. Appuhamy, and M. D. Hanigan. 2010. M-tor independent model of protein synthesis regulation by essential amino acids in mammary epithelial cells. Page 247 in Energy and protein metabolism and nutrition. M. G. Crovetto, ed. Wageningen Academic, Parma, Italy.

Bai, X., D. Ma, A. Liu, X. Shen, Q. J. Wang, Y. Liu, and Y. Jiang. 2007. Rheb activates mtor by antagonizing its endogenous inhibitor, fkbp38. Science 318(5852):977-980.

Baker, D. H. 1996. Advances in amino acid nutrition and metabolism of swine and poultry. Pages 41-52 in Nutrient managment of food animals to enhance and protect the environment. E. T. Kornegay, ed. CRC Lewis, Boca Raton, FL.

Basu, A. and D. Chowdhury. 2007. Modeling protein synthesis from a physicist's perspective: A toy model. Am. J. Phys. 75(10):931-937.

Baumrucker, C. R. 1985. Amino acid transport systems in bovine mammary tissue1. J. Dairy Sci. 68(9):2436-2451.

Bellacosa, A., T. O. Chan, N. N. Ahmed, K. Datta, S. Malstrom, D. Stokoe, F. McCormick, J. Feng, and P. Tsichlis. 1998. Akt activation by growth factors is a multiple-step process: The role of the ph domain. Oncogene 17(3):313-325.

Bequette, B. J., F. R. C. Backwell, J. C. MacRae, G. E. Lobley, L. A. Crompton, J. A. Metcalf, and J. D. Sutton. 1996. Effect of intravenous amino acid infusion on leucine oxidation across the mammary gland of the lactating goat. J. Dairy Sci. 79(12):2217-2224.

Bequette, B. J., M. D. Hanigan, A. G. Calder, C. K. Reynolds, G. E. Lobley, and J. C. MacRae. 2000. Amino acid exchange by the mammary gland of lactating goats when histidine limits milk production. J. Dairy Sci. 83(4):765-775.
Boguhn, J., H. Kluth, and M. Rodehutscord. 2006. Effect of total mixed ration composition on amino acid profiles of different fractions of ruminal microbes in vitro. J. Dairy Sci. 89(5):1592-1603.
Cant, J. P. and B. W. McBride. 1995. Mathematical analysis of the relationship between blood flow and uptake of nutrients in the mammary glands of a lactating cow. J. Dairy Res. 62(03):405-422.
Cant, J. P., D. R. Trout, F. Qiao, and B. W. McBride. 2001. Milk composition responses to unilateral arterial infusionof complete and histidine-lacking amino acid mixtures to the mammary glands of cows. J. Dairy Sci. 84(5):1192-1200.
Cant, J. P., R. Berthiaume, H. Lapierre, P. H. Luimes, B. W. McBride, and D. Pacheco. 2003. Responses of the bovine mammary glands to absorptive supply of single amino acids. Can. J. Anim. Sci. 83(3):341-355.
Caraviello, D. Z., K. A. Weigel, P. M. Fricke, M. C. Wiltbank, M. J. Florent, N. B. Cook, K. V. Nordlund, N. R. Zwald, and C. L. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large us commercial farms. J. Dairy Sci. 89(12):4723-4735.
Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows1. J. Dairy Sci. 75(8):2304-2323.
Clark, R. M., P. T. Chandler, and C. S. Park. 1978. Limiting amino acids for milk protein synthesis by bovine mammary cells in culture1. J. Dairy Sci. 61(4):408-413.
Cyriac, J., A. G. Rius, M. L. McGilliard, R. E. Pearson, B. J. Bequette, and M. D. Hanigan. 2008. Lactation performance of mid-lactation dairy cows fed ruminally degradable protein at concentrations lower than national research council recommendations. J. Dairy Sci. 91(12):4704-4713.
Davis, T. A., M. L. Fiorotto, D. G. Burrin, P. J. Reeds, H. V. Nguyen, P. R. Beckett, R. C. Vann, and P. M. J. O'Connor. 2002. Stimulation of protein synthesis by both insulin and amino acids is unique to skeletal muscle in neonatal pigs. Am. J. Physiol. Endocrinol. Metab. 282(4):E880-890.
Delgado-Elorduy, A., C. B. Theurer, J. T. Huber, A. Alio, O. Lozano, M. Sadik, P. Cuneo, H. D. De Young, I. J. Simas, J. E. P. Santos, L. Nussio, C. Nussio, K. E. Webb Jr, and H. Tagari. 2002. Splanchnic and mammary nitrogen metabolism by dairy cows fed dry-rolled or steam-flaked sorghum grain. J. Dairy Sci. 85(1):148-159.
Doepel, L., D. Pacheco, J. J. Kennelly, M. D. Hanigan, I. F. López, and H. Lapierre. 2004. Milk protein synthesis as a function of amino acid supply. J. Dairy Sci. 87(5):1279-1297.
Doepel, L. and H. Lapierre. 2010. Changes in production and mammary metabolism of dairy cows in response to essential and nonessential amino acid infusions. J. Dairy Sci. 93(7):3264-3274.
Doppler, W., B. Groner, and R. K. Ball. 1989. Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat beta-casein gene

promoter constructs in a mammary epithelial cell line. Proceedings of the National Academy of Sciences 86(1):104-108.
Dos, D. S., S. M. Ali, D.-H. Kim, D. A. Guertin, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2004. Rictor, a novel binding partner of mtor, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr. Biol. 14(14):1296-1302.
Drummond, M. J., E. L. Glynn, C. S. Fry, K. L. Timmerman, E. Volpi, and B. B. Rasmussen. 2010. An increase in essential amino acid availability upregulates amino acid transporter expression in human skeletal muscle. Am. J. Physiol. Endocrinol. 298(5):E1011-E1018.
Dunlop, E. A. and A. R. Tee. 2009. Mammalian target of rapamycin complex 1: Signalling inputs, substrates and feedback mechanisms. Cell. Signal. 21(6):827-835.
El-Haroun, E. R., D. P. Bureau, and J. P. Cant. 2010. A mechanistic model of nutritional control of protein synthesis in animal tissues. J. Theor. Biol. 262(2):361-369.
Elf, J., D. Nilsson, T. Tenson, and M. n. Ehrenberg. 2003. Selective charging of trna isoacceptors explains patterns of codon usage. Science 300(5626):1718-1722.
Erasmus, L. J., P. M. Botha, C. W. Cruywagen, and H. H. Meissner. 1994. Amino acid profile and intestinal digestibility in dairy cows of rumen-undegradable protein from various feedstuffs1. J. Dairy Sci. 77(2):541-551.
Gan, X., J. Wang, B. Su, and D. Wu. 2011. Evidence for direct activation of mtorc2 kinase activity by phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. 286(13):10998-11002.
Guinard, J. and H. Rulquin. 1994. Effects of graded amounts of duodenal infusions of lysine on the mammary uptake of major milk precursors in dairy cows. J. Dairy Sci. 77(12):3565-3576.
Guinard, J. and H. Rulquin. 1995. Effects of graded amounts of duodenal infusions of methionine on the mammary uptake of major milk precursors in dairy cows. J. Dairy Sci. 78(10):2196-2207.
Gwinn, D. M., D. B. Shackelford, D. F. Egan, M. M. Mihaylova, A. Mery, D. S. Vasquez, B. E. Turk, and R. J. Shaw. 2008. Ampk phosphorylation of raptor mediates a metabolic checkpoint. Mol. Cell 30(2):214-226.
Han, Jung M., Seung J. Jeong, Min C. Park, G. Kim, Nam H. Kwon, Hoi K. Kim, Sang H. Ha, Sung H. Ryu, and S. Kim. 2012. Leucyl-trna synthetase is an intracellular leucine sensor for the mtorc1-signaling pathway. Cell 149(2):410-424.
Hanigan, M. D., C. C. Calvert, E. J. DePeters, B. L. Reis, and R. L. Baldwin. 1992. Kinetics of amino acid extraction by lactating mammary glands in control and sometribove-treated holstein cows. J. Dairy Sci. 75(1):161-173.
Hanigan, M. D., J. P. Cant, D. C. Weakley, and J. L. Beckett. 1998. An evaluation of postabsorptive protein and amino acid metabolism in the lactating dairy cow. J. Dairy Sci. 81(12):3385-3401.
Hanigan, M. D., J. France, L. A. Crompton, and B. J. Bequette. 2000. Evaluation of a representation of the limiting amino acid theory for milk protein synthesis. in Modelling nutrient utilization in farm animals. J. P. McNamara, J. France, and D. E. Beever, ed. CABI, Wallingford.

Hanigan, M. D., L. A. Crompton, J. A. Metcalf, and J. France. 2001. Modelling mammary metabolism in the dairy cow to predict milk constituent yield, with emphasis on amino acid metabolism and milk protein production: Model construction. J. Theor. Biol. 213(2):223-239.

Hanigan, M. D., L. A. Crompton, B. J. Bequette, J. A. Mills, and J. France. 2002. Modelling mammary metabolism in the dairy cow to predict milk constituent yield, with emphasis on amino acid metabolism and milk protein production: Model evaluation. J. Theor. Biol. 217(3):311-330.

Hanigan, M. D., L. A. Crompton, C. K. Reynolds, D. Wray-Cahen, M. A. Lomax, and J. France. 2004a. An integrative model of amino acid metabolism in the liver of the lactating dairy cow. J. Theor. Biol. 228(2):271-289.

Hanigan, M. D., C. K. Reynolds, D. J. Humphries, B. Lupoli, and J. D. Sutton. 2004b. A model of net amino acid absorption and utilization by the portal-drained viscera of the lactating dairy cow. J. Dairy Sci. 87(12):4247-4268.

Haque, M. N., H. Rulquin, A. Andrade, P. Faverdin, J. L. Peyraud, and S. Lemosquet. 2012. Milk protein synthesis in response to the provision of an "ideal" amino acid profile at 2 levels of metabolizable protein supply in dairy cows. J. Dairy Sci. 95(10):5876-5887.

Haque, M. N., H. Rulquin, and S. Lemosquet. 2013. Milk protein responses in dairy cows to changes in postruminal supplies of arginine, isoleucine, and valine. J. Dairy Sci. 96(1):420-430.

Hardie, D. G. 2004. The amp-activated protein kinase pathway - new players upstream and downstream. J. Cell Sci. 117(23):5479-5487.

Hristov, A. N., R. P. Etter, J. K. Ropp, and K. L. Grandeen. 2004a. Effect of dietary crude protein level and degradability on ruminal fermentation and nitrogen utilization in lactating dairy cows. J. Anim. Sci. 82(11):3219-3229.

Hristov, A. N., W. J. Price, and B. Shafii. 2004b. A meta-analysis examining the relationship among dietary factors, dry matter intake, and milk and milk protein yield in dairy cows. J. Dairy Sci. 87(7):2184-2196.

Inoki, K., Y. Li, T. Zhu, J. Wu, and K.-L. Guan. 2002. Tsc2 is phosphorylated and inhibited by akt and suppresses mtor signalling. Nat. Cell Biol. 4(9):648-657.

Inoki, K., Y. Li, T. Xu, and K.-L. Guan. 2003a. Rheb gtpase is a direct target of tsc2 gap activity and regulates mtor signaling. Genes & Development 17(15):1829-1834.

Inoki, K., T. Zhu, and K.-L. Guan. 2003b. Tsc2 mediates cellular energy response to control cell growth and survival. Cell 115(5):577-590.

Ipharraguerre, I. R. and J. H. Clark. 2005. Varying protein and starch in the diet of dairy cows. Ii. Effects on performance and nitrogen utilization for milk production. J. Dairy Sci. 88(7):2556-2570.

Ipharraguerre, I. R., J. H. Clark, and D. E. Freeman. 2005. Varying protein and starch in the diet of dairy cows. I. Effects on ruminal fermentation and intestinal supply of nutrients. J. Dairy Sci. 88(7):2537-2555.

Kilberg, M. S., Y.-X. Pan, H. Chen, and V. Leung-Pineda. 2005. Nutritional control of gene expression: How mammalian cells respond to amino acid limitation. Annu. Rev. Nutr. 25(1):59-85.

Korhonen, M., A. Vanhatalo, T. Varvikko, and P. Huhtanen. 2000. Responses to graded postruminal doses of histidine in dairy cows fed grass silage diets. J. Dairy Sci. 83(11):2596-2608.
Kronfeld, D., F. Raggi, and C. Ramberg. 1968. Mammary blood #ow and ketone body metabolism in normal, fasted and ketotic cows. Am. J. Physiol. 215(1):218.
Lapierre, H. and G. E. Lobley. 2001. Nitrogen recycling in the ruminant: A review. J. Dairy Sci. 84(Supplement 1):E223-E236.
Lapierre, H., R. Berthiaume, G. Raggio, M. C. Thivierge, L. Doepel, D. Pacheco, P. Dubreuil, and G. E. Lobley. 2005. The route of absorbed nitrogen into milk protein. Anim. Sci. 80(01):11-22.
Lapierre, H., D. R. Ouellet, R. Berthiaume, R. Martineau, G. Holtrop, and G. E. Lobley. 2008. Distribution of 15n in amino acids during 15n-leucine infusion: Impact on the estimation of endogenous flows in dairy cows. J. Dairy Sci. 91(7):2702-2714.
Lapierre, H., C. E. Galindo, S. Lemosquet, I. Ortigues-Marty, L. Doepel, and D. R. Ouellet. 2010. Protein supply, glucose kinetics and milk yield in dairy cows. Pages 275-286 in Energy and protein metabolism and nutrition. G. M. Crovetto, ed. Wageningen Academic Publishers, Wageningen.
Lemay, D., M. Neville, M. Rudolph, K. Pollard, and J. German. 2007. Gene regulatory networks in lactation: Identification of global principles using bioinformatics. BMC Syst. Biol. 1(1):56.
Lobley, G. E., D. M. Bremner, and G. Zuur. 2000a. Effects of diet quality on urea fates in sheep as assessed by refined, non-invasive [nn] urea kinetics. Br. J. Nutr. 84(04):459-468.
Lobley, G. E., G. D. Milano, and J. G. VAN DER Walt. 2000b. The liver: Integrator of nitrogen metabolism. in Ruminant physiology: Digestion, metabolism, growth, and reproduction. P. B. Cronjé, ed. CABI Publishing, New York.
Lomax, M. A. and G. D. Baird. 1983. Blood flow and nutrient exchange across the liver and gut of the dairy cow. Br. J. Nutr. 49(03):481-496.
Mackenzie, B. and J. Erickson. 2004. Sodium-coupled neutral amino acid (system n/a) transporters of the slc38 gene family. Pflugers Arch. 447(5):784-795.
Mackle, T. R., D. A. Dwyer, K. L. Ingvartsen, P. Y. Chouinard, D. A. Ross, and D. E. Bauman. 2000. Effects of insulin and postruminal supply of protein on use of amino acids by the mammary gland for milk protein synthesis. J. Dairy Sci. 83(1):93-105.
MacRae, J. C., L. A. Bruce, D. S. Brown, and A. G. Calder. 1997. Amino acid use by the gastrointestinal tract of sheep given lucerne forage. American Journal of Physiology - Gastrointestinal and Liver Physiology 273(6):G1200-G1207.
Mahoney, S. J., J. M. Dempsey, and J. Blenis. 2009. Chapter 2 cell signaling in protein synthesis: Ribosome biogenesis and translation initiation and elongation. Pages 53-107 in Progress in molecular biology and translational science. Vol. Volume 90. W. B. H. John, ed. Academic Press.
Menzies, K., C. Lefèvre, K. Macmillan, and K. Nicholas. 2009. Insulin regulates milk protein synthesis at multiple levels in the bovine mammary gland. Functional & Integrative Genomics 9(2):197-217.

Metcalf, J. A., L. A. Crompton, D. Wray-Cahen, M. A. Lomax, J. D. Sutton, D. E. Beever, J. C. MacRae, B. J. Bequette, F. R. C. Backwell, and G. E. Lobley. 1996a. Responses in milk constituents to intravascular administration of two mixtures of amino acids to dairy cows. J. Dairy Sci. 79(8):1425-1429.

Metcalf, J. A., D. Wray-Cahen, E. E. Chettle, J. D. Sutton, D. E. Beever, L. A. Crompton, J. C. Macrae, B. J. Bequette, and F. R. C. Backwell. 1996b. The effect of dietary crude protein as protected soybean meal on mammary metabolism in the lactating dairy cow. J. Dairy Sci. 79(4):603-611.

Mitchell, H. H. and R. J. Block. 1946. Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem. 163(3):599-620.

Moshel, Y., R. E. Rhoads, and I. Barash. 2006. Role of amino acids in translational mechanisms governing milk protein synthesis in murine and ruminant mammary epithelial cells. J. Cell. Biochem. 98(3):685-700.

Noftsger, S. and N. R. St-Pierre. 2003. Supplementation of methionine and selection of highly digestible rumen undegradable protein to improve nitrogen efficiency for milk production1. J. Dairy Sci. 86(3):958-969.

NRC. 1989. Nutrient requirements of dairy cattle. 6th ed. National Academy Press, Washington, DC.

NRC. 2001. Nutrient requirements of dairy cattle. 7th ed. National Academy Press, Washington, DC.

Olmos Colmenero, J. J. and G. A. Broderick. 2006. Effect of dietary crude protein concentration on milk production and nitrogen utilization in lactating dairy cows. J. Dairy Sci. 89(5):1704-1712.

Park, C. S., P. T. Chandler, and R. M. Clark. 1976. Optimum amino acid complement for protein synthesis by rat mammary cells in tissue culture1. J. Dairy Sci. 59(10):1758-1763.

Piepenbrink, M. S. and D. J. Schingoethe. 1998. Ruminal degradation, amino acid composition, and estimated intestinal digestibilities of four protein supplements1. J. Dairy Sci. 81(2):454-461.

Raggio, G., D. Pacheco, R. Berthiaume, G. E. Lobley, D. Pellerin, G. Allard, P. Dubreuil, and H. Lapierre. 2004. Effect of level of metabolizable protein on splanchnic flux of amino acids in lactating dairy cows. J. Dairy Sci. 87(10):3461-3472.

Raggio, G., G. E. Lobley, R. Berthiaume, D. Pellerin, G. Allard, P. Dubreuil, and H. Lapierre. 2007. Effect of protein supply on hepatic synthesis of plasma and constitutive proteins in lactating dairy cows. J. Dairy Sci. 90(1):352-359.

Reynolds, C. K. and N. B. Kristensen. 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: An asynchronous symbiosis. J. Anim. Sci. 86(14 suppl):E293-E305.

Rius, A. G., J. A. D. R. N. Appuhamy, J. Cyriac, D. Kirovski, O. Becvar, J. Escobar, M. L. McGilliard, B. J. Bequette, R. M. Akers, and M. D. Hanigan. 2010. Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. J. Dairy Sci. 93(7):3114-3127.

Rodríguez-Prado, M., S. Calsamiglia, and A. Ferret. 2004. Effects of fiber content and particle size of forage on the flow of microbial amino acids from continuous culture fermenters*. J. Dairy Sci. 87(5):1413-1424.

Røjen, B. A., M. Larsen, and N. B. Kristensen. 2012. Effect of abomasal infusion of oligofructose on portal-drained visceral ammonia and urea-nitrogen fluxes in lactating holstein cows. J. Dairy Sci. 95(12):7248-7260.
Rulquin, H., S. Rigout, S. Lemosquet, and A. Bach. 2004. Infusion of glucose directs circulating amino acids to the mammary gland in well-fed dairy cows. J. Dairy Sci. 87(2):340-349.
Sancak, Y., L. Bar-Peled, R. Zoncu, A. L. Markhard, S. Nada, and D. M. Sabatini. 2010. Ragulator-rag complex targets mtorc1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141(2):290-303.
Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and M. M. A. Mesbah. 1992a. Amino acid limitation and flow to duodenum at four stages of lactation. 1. Sequence of lysine and methionine limitation. J. Dairy Sci. 75(12):3486-3502.
Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and V. M. Olson. 1992b. Amino acid limitation and flow to the duodenum at four stages of lactation. 2. Extent of lysine limitation. J. Dairy Sci. 75(12):3503-3518.
Sharma, A. K. and D. Chowdhury. 2011. Stochastic theory of protein synthesis and polysome: Ribosome profile on a single mrna transcript. J. Theor. Biol. 289(0):36-46.
Shennan, D. B., I. D. Millar, and D. T. Calvert. 1997. Mammary-tissue amino acid transport systems. Proc. Nutr. Soc. 56(1A):177-191.
St-Pierre, N. R. 2012. The costs of nutrients, comparison of feedstuffs prices and the current dairy situation. in Buckeye News. The Ohio State University Extension, http://dairy.osu.edu/bdnews/bdnews.html.
Stewart, K. W., G. J. S. Cooper, and S. R. Davis. 2009. Coordination of mammary metabolism and blood flow after refeeding in rats. J. Dairy Sci. 92(4):1543-1553.
Storm, E., D. S. Brown, and E. R. Ørskov. 1983. The nutritive value of rumen micro-organisms in ruminants. Br. J. Nutr. 50(02):479-485.
Tagari, H., K. Webb Jr, B. Theurer, T. Huber, D. DeYoung, P. Cuneo, J. E. P. Santos, J. Simas, M. Sadik, A. Alio, O. Lozano, A. Delgado-Elorduy, L. Nussio, C. M. M. Bittar, and F. Santos. 2008. Mammary uptake, portal-drained visceral flux, and hepatic metabolism of free and peptide-bound amino acids in cows fed steam-flaked or dry-rolled sorghum grain diets. J. Dairy Sci. 91(2):679-697.
Toerien, C. A., D. R. Trout, and J. P. Cant. 2010. Nutritional stimulation of milk protein yield of cows is associated with changes in phosphorylation of mammary eukaryotic initiation factor 2 and ribosomal s6 kinase 1. J. Nutr. 140(2):285-292.
Tuller, T., A. Carmi, K. Vestsigian, S. Navon, Y. Dorfan, J. Zaborske, T. Pan, O. Dahan, I. Furman, and Y. Pilpel. 2010. An evolutionarily conserved mechanism for controlling the efficiency of protein translation. Cell 141(2):344-354.
Vander Haar, E., S.-i. Lee, S. Bandhakavi, T. J. Griffin, and D.-H. Kim. 2007. Insulin signalling to mtor mediated by the akt/pkb substrate pras40. Nat. Cell Biol. 9(3):316-323.
Vanhatalo, A., P. Huhtanen, V. Toivonen, and T. Varvikko. 1999. Response of dairy cows fed grass silage diets to abomasal infusions of histidine alone or in combinations with methionine and lysine. J. Dairy Sci. 82(12):2674-2685.

Vanhatalo, A., T. Varvikko, and P. Huhtanen. 2003. Effects of casein and glucose on responses of cows fed diets based on restrictively fermented grass silage. J. Dairy Sci. 86(10):3260-3270.
Vinod, P. K. U. and K. V. Venkatesh. 2009. Quantification of the effect of amino acids on an integrated mtor and insulin signaling pathway. Molecular BioSystems 5(10):1163-1173.
von Liebig, J. 1863. The natural laws of husbandry. Walton & Maberly, London.
Wang, L., T. E. Harris, R. A. Roth, and J. C. Lawrence. 2007. Pras40 regulates mtorc1 kinase activity by functioning as a direct inhibitor of substrate binding. J. Biol. Chem. 282(27):20036-20044.
Whitelaw, F. G., J. S. Milne, E. R. Ørskov, and J. S. Smith. 1986. The nitrogen and energy metabolism of lactating cows given abomasal infusions of casein. Br. J. Nutr. 55(03):537-556.
Wolfe, A. H. and J. A. Patz. 2002. Reactive nitrogen and human health:Acute and long-term implications. Ambio 31(2):120-125.
Wu, Z., C. E. Polan, and R. J. Fisher. 1997. Adequacy of amino acids in diets fed to lactating dairy cows. J. Dairy Sci. 80(8):1713-1721.

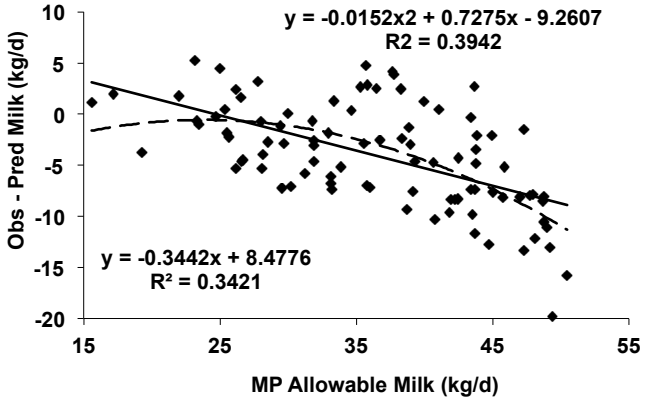
y = -0.0152x2 + 0.7275x - 9.2607
R2 = 0.3942
y = -0.3442x + 8.4776
R² = 0.3421
Obs - Pred Milk (kg/d)
MP Allowable Milk (kg/d)
10
5
0
-5
-10
-15
-20
15
25
35
45
55

Figure 2.1. Milk (kg/d) residual errors (observed - predicted) as function of MP allowable milk (kg/d) supply.
Points represent individual residuals, the solid line represents the predicted linear function of those residuals, and the dashed line represents a predicted quadratic function. Derived from NRC, 2001.

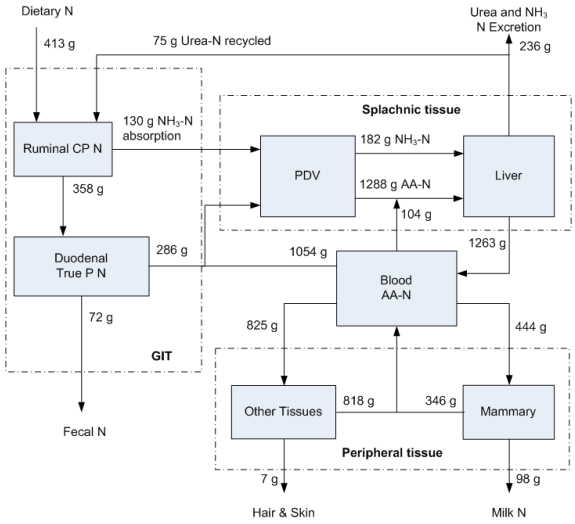
Dietary N
413 g
75 g Urea-N recycled
Urea and NH3 N Excretion
236 g
Splachnic tissue
Ruminal CP N
130 g NH3-N absorption
PDV
182 g NH3-N
Liver
1288 g AA-N
358 g
104 g
1263 g
Duodenal True P N
286 g
1054 g
Blood AA-N
72 g
825 g
444 g
GIT
Other Tissues
818 g
346 g
Mammary
Fecal N
Peripheral tissue
7 g
98 g
Hair & Skin
Milk N

Figure 2.2. Nitrogen flux diagram derived from Hanigan et al., 2004b.
Solid boxes represent pools, open boxes represent compartments and numbers indicate fluxes (g N/d). GIT: gastro-intestinal-tract. Duodenal AA flux was obtained from NRC, 2001. Urine and fecal N excretion, and other tissue N losses were calculated by difference.

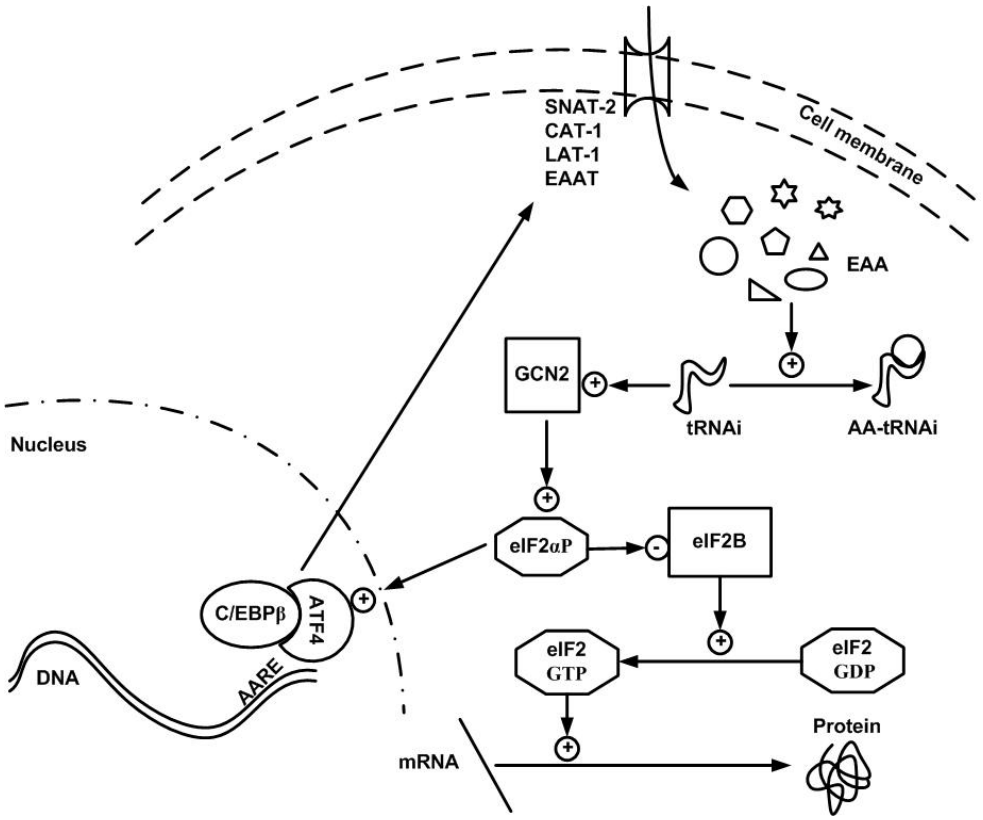
SNAT-2
CAT-1
LAT-1
EAAT
Cell membrane
EAA
GCN2
tRNAi
AA-tRNAi
Nucleus
eIF2αP
eIF2B
C/EBPβ
ATF4
DNA
AARE
eIF2
GTP
eIF2
GDP
Protein
mRNA

Figure 2.3. Schematic representation of the general AA response pathway involved in protein translation and AA transporter gene expression regulation in mammalian cells. ATF4 = activating transcription factor 4; C/EBP ß = CCAAT enhancer binding protein; EAA = essential AA; eIF2αP = eukaryotic initiation factor 2 alpha subunit phosphorylated in the Ser51 phospho site; eIF2B = eukaryotic initiation factor 2 B; GCN2 = general control non-derepressible 2; mRNA = messenger RNA; tRNAi = individual transfer RNAs.

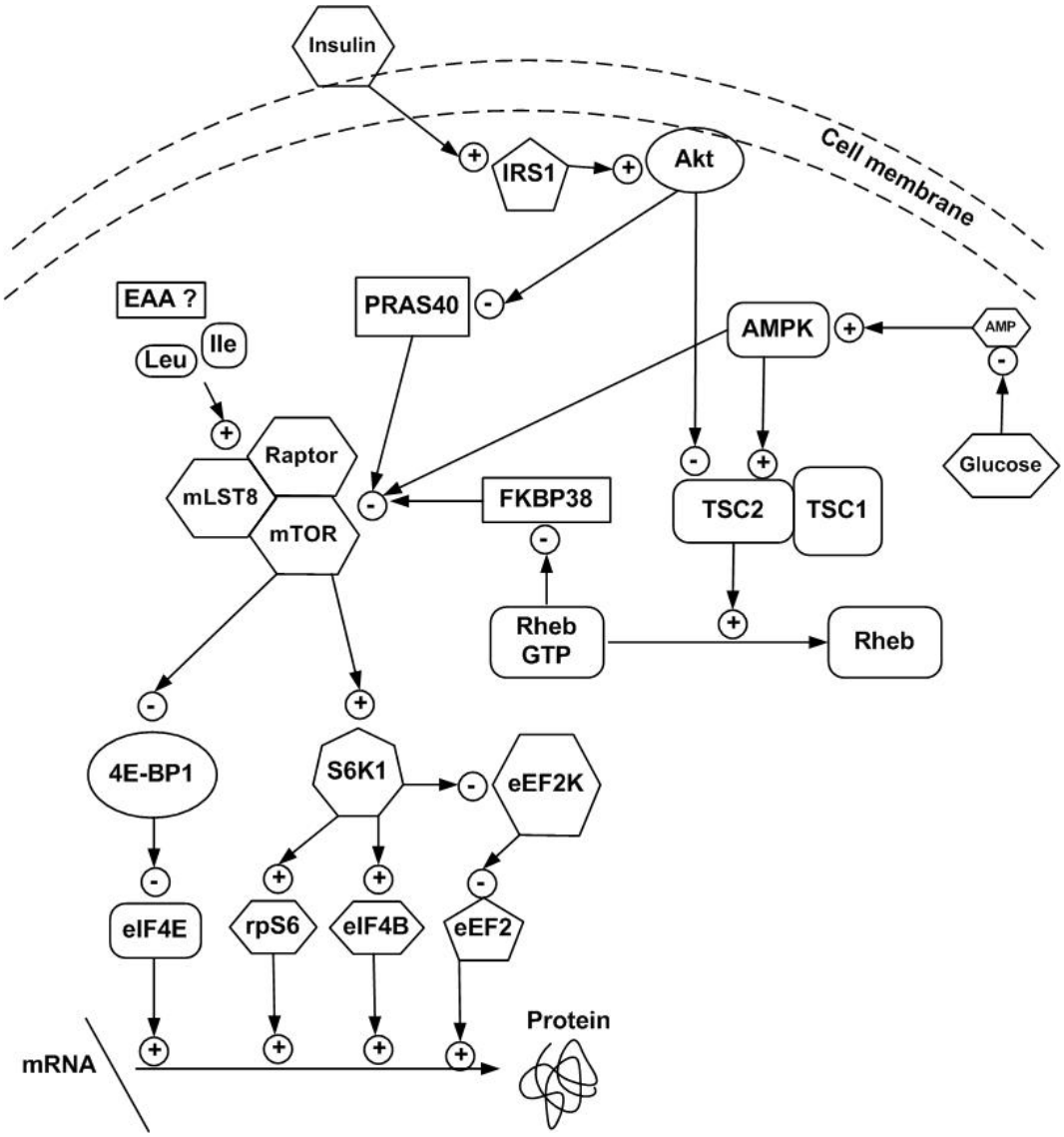
Insulin
Cell membrane
IRS1
Akt
PRAS40
EAA ?
Leu
Ile
AMPK
AMP
Glucose
Raptor
mLST8
mTOR
FKBP38
TSC2
TSC1
Rheb GTP
Rheb
4E-BP1
S6K1
eEF2K
eIF4E
rpS6
eIF4B
eEF2
mRNA
Protein

Figure 2.4. mTORC1 translation regulation pathway.
Depicted are the effects of insulin, AMP, and identified AA, as well as other potential essential AA, on mTORC1 pathway. There are also represented several effects of the mTORC1 downstream protein on translation regulation. 4EBP1 = eukaryotic initiation factor 4 E binding protein 1; AMPK = AMP kinase; Akt = protein kinase B; EAA = essential AA; eEF2 = eukaryotic elongation factor 2; eEF2K = eukaryotic elongation factor 2 kinase; eIF4E = eukaryotic initiation factor 4 E; FKBP38 = FK506 binding protein 38; IRS1 = insulin receptor substrate 1; mLST8 = mammalian lethal with SEC3 protein 8; mRNA = messenger RNA; mTORC1 = mammalian target of rapamycin complex 1; PRAS40 = proline-rich Akt substrate 40 kDa; Rheb = Ras homolog enriched in brain; rpS6 = ribosomal protein S6; TSC = tuberous sclerosis complex; S6K1 = ribosomal protein S6 kinase 1.

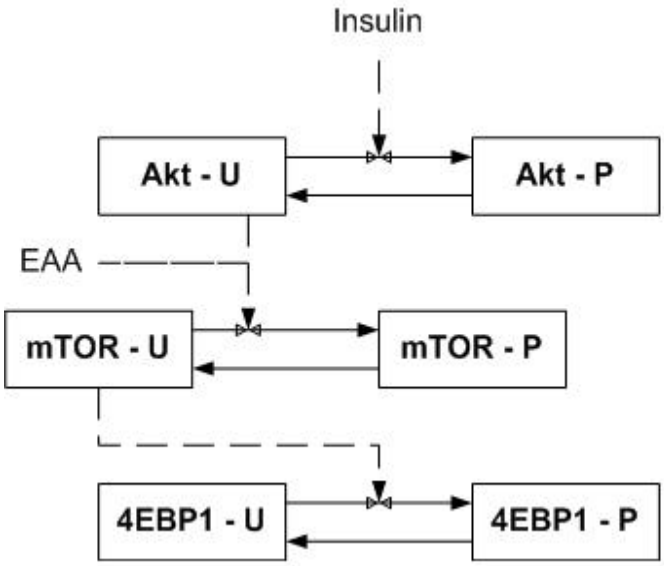
Insulin
Akt - U
Akt - P
EAA
mTOR - U
mTOR - P
4EBP1 - U
4EBP1 - P

Figure 2.5. Schematic of the mTORC1 signaling pathway model derived from Appuhamy and Hanigan (2010).
Solid arrows represent fluxes, dashed arrows represent regulatory effects on the fluxes and solid boxes represent protein pools. 4EBP1 = eukaryotic initiation factor 4 E binding protein 1; Akt = protein kinase B; EAA = essential AA; mTORC1 = mammalian target of rapamycin complex 1; U = unphosphorylated; P = phosphorylated.

CHAPTER 3: Ile, Leu, Met and Thr effects on mTOR signaling in mammary tissue slices

ABSTRACT

Improved representation of post-absorptive N metabolism in lactating dairy cows requires a better understanding of protein synthesis regulation in the mammary gland. This study aimed to determine the quantitative effects of Ile, Leu, Met, and Thr on the phosphorylation state of signaling proteins that regulate protein synthesis. The experiment utilized a composite design with a central point, 2 axial points per AA, and a complete 2^4 factorial. All of the other AA were at the concentrations in DMEM. The experiment was replicated with tissues from 5 lactating cows. Mammary tissue slices (0.12 ± 0.02 g) were incubated for 4 h. Total and site-specific phosphorylated mammalian target of rapamycin (mTOR; Ser2448), eukaryotic elongation factor (eEF) 2 (Thr56), ribosomal protein (rp) S6 (Ser235/236), and eukaryotic initiation factor (eIF) 2α (Ser51) were determined by western immunoblotting. Intracellular concentrations of the four AA studied responded linearly to media supply ($P \leq 0.001$). Addition of Ile, Leu, Met, or Thr had no effect on eIF2α phosphorylation ($P < 0.05$). Isoleucine ($P = 0.03$) and Thr ($P = 0.04$) positively affected mTOR phosphorylation. However, the two had an antagonistic relationship (Ile×Thr, $P = 0.05$). Similarly, Ile linearly ($P = 0.003$) increased rpS6 phosphorylation, and Thr inhibited the Ile effect (Ile×Thr, $P = 0.007$). In addition, eEF2 phosphorylation was linearly decreased by Ile ($P = 0.02$) and Leu ($P = 0.002$). Threonine curvilinearly decreased eEF2 phosphorylation (Thr×Thr, $P = 0.03$). Ile and Leu

negatively interacted on eEF2 (Ile×Leu, $P = 0.02$) and Thr (Leu×Thr, $P = 0.07$) tended to inhibit Leu effects on eEF2.

Keywords: essential AA, mTOR, translation regulation, mammary gland.

INTRODUCTION

Among the dairy industry pollutants, N excretion is one of the major concerns because of its impact on air and water quality, ecosystem biodiversity, and human health (Wolfe and Patz, 2002). There are approximately 9M dairy cattle in the US (Livestock, Dairy, and Poultry Outlook: August 2012, LDPM-218, Dairy Economic Research Service, USDA). A representative survey across dairy nutritionist estimated that average diets contain 17.8 ± 0.1% CP (Caraviello et al., 2006). A meta-analysis of 846 experimental diets determined that with that dietary CP content, only one quarter of dietary N is recovered in product. Therefore, the dairy industry releases about 1.3 million metric tons of dietary N per year.

Among N losses, post-absorptive catabolism of AA is the most significant source because it accounts for approximately half of the excreted N and two-thirds of the absorbed non-ammonia N (Hanigan et al., 2004). Amino acid catabolism by splanchnic tissues is proportional to supply (Raggio et al., 2004) but only a small proportion of dietary AA is captured in the first pass (MacRae et al., 1997). Most of the AA catalyzed by splanchnic tissues are recycled from peripheral circulation. Therefore, if mammary extraction of AA increased, recycling and splanchnic catabolism would decrease. Contrary to splanchnic tissues, mammary utilization of AA is not a linear function of supply but decreases as supply increases (Hanigan et al., 2000, Lapierre et al., 2010, Whitelaw et al., 1986). Hence, low CP diets should have higher efficiency than high CP diets. However, at a given level of dietary CP, different AA profiles can yield different levels of milk protein and thus vary N post-absorptive efficiency for milk protein (Haque et al., 2012).

Amino acids not only serve as substrates for protein synthesis but also function as signaling molecules that regulate the process (Kimball, 2002). Specific AA are known to affect translation initiation and elongation rates through two main pathways: the integrated stress response (**ISR**) and the mammalian target of rapamycin (mTOR). The former regulates methionyl-tRNA recruitment to the 40 S ribosomal subunit that is catalyzed by eukaryotic initiation factor (eIF) 2 in a GTP-dependent manner. Phosphorylated eIF2α blocks the release of GDP upon hydrolysis from eIF2 and stops further translation initiation. Four distinct eIF2α kinases that respond to different stress signals have been identified. Particularly, depletion of AA indirectly activates general control non-derepressable (GCN) 2, which binds deacylated tRNA. Among others, this pathway is active in mammary epithelial cells. In MAC-T cells, removing all EAA from Dulbecco's Modified Eagle Medium (DMEM) medium increased eIF2α phosphorylation (Appuhamy et al., 2011a). Meanwhile, supplementing His, Phe, and Val back to medium reduced the phosphorylation level (Arriola Apelo et al., 2010). Similarly, in mammary tissue slices, subtracting all EAA from DMEM significantly increased eIF2α phosphorylation (Appuhamy et al., 2012). However, in that study, individual EAA had only numerical effects eIF2α phosphorylation.

The mTOR pathway is highly conserved, being expressed in species from avian (Metayer-Coustard et al., 2010) to fish (Lansard et al., 2011) and mammals, and in different tissues (Murgas Torrazza et al., 2010). It revolves around mTOR complex 1 (mTORC1). This complex contains mTOR, a serine-threonine kinase, Raptor, and mLST8 (Mahoney et al., 2009). In complex 1, mTOR phosphorylates eIF4 binding protein 1 (4E-BP1) and ribosomal protein S6 (rpS6) kinase 1 (S6K1). When

unphosphorylated, 4E-BP1 antagonizes eIF4G binding to eIF4E and inhibits translation (Mahoney et al., 2009). Ribosomal protein S6 kinase1 phosphorylates rpS6 and eIF4B to promote translation initiation. Another target of S6K1 is eukaryotic elongation factor (eEF) 2 kinase. This kinase is inhibited by phosphorylation, resulting in activation of eEF2 and increased elongation rate (Dunlop and Tee, 2009).

Signaling molecules that activate mTORC1 vary between tissues. Skeletal muscle is the most responsive. In neonatal pigs, skeletal muscle responds to insulin, EAA, and Leu (Davis et al., 2002, Escobar et al., 2006). Liver responds to EAA, but not to insulin or Leu (Davis et al., 2002, Murgas Torrazza et al., 2010). In bovine mammary epithelial cells, deprivation of all AA or Leu affected S6K1 and 4E-BP1 phosphorylation and fractional synthesis rate of ß-lactoglobulin (Moshel et al., 2006). Similarly, in mammary tissue EAA and Ile or Leu alone increased mTOR phosphorylation and casein fractional synthesis rate (CFSR), (Appuhamy et al., 2011a, 2012). Insulin activated the Akt pathway (Akt and IRS1), and increased phosphorylation of mTOR, and downstream proteins S6K1, and 4EBP1, in MAC-T cells (Appuhamy et al., 2011a).

Individual AA have different effects on signaling proteins and CFSR. A reasonable strategy to increase post-absorptive N efficiency would be to decrease current levels of dietary N and supplement with those specific AA that have the largest effect on translation regulation and protein synthesis. That would increase the mammary glands' demand and extraction of AA, and reduce recycling to splanchnic tissues (Hanigan et al., 1998). To incorporate this strategy in nutrient requirement systems, it is necessary to understand the quantitative effect of AA on cell signaling and CFSR, as well as identification of interactions. Therefore, this study aimed to determine the effects of the

four EAA (Ile, Leu, Met, and Thr) known to stimulate CFSR (Appuhamy et al., 2012) on phosphorylation of signaling proteins that regulate translation initiation and elongation in mammary tissue slices.

METHODS

Tissue Collection

Mammary tissue was obtained from 5 multiparous dairy cows belonging to the Virginia Tech dairy herd. Cows (Holstein or Holstein x Jersey crossbred) were removed from feed 12 h, and milked 2 h before slaughter, which was performed at the Virginia Tech Meat Laboratory Center. Milk from each quarter was tested for somatic cell count prior to slaughter to ensure the harvested tissue was not from an infected quarter. Mammary tissue from the upper part of rear quarters was excised and transported in ice-cold PBS to the laboratory where tissue slices were prepared within two hours of slaughter.

Incubation

A base medium was prepared the day before the experiment from DMEM devoid of EAA (Gibco Invitrogen, custom formula no. 08-5072EL) and containing 3.7 mmol non-EAA, 0.7 mmol $_{L}$-arginine, 0.15 mmol $_{L}$-histidine, 0.5 mmol $_{L}$-lysine, 0.45 mmol $^{2}H_{5}$-$_{L}$-phenylalanine, 0.04 mmol $_{L}$-tryptophan, 0.45 mmol $_{L}$-valine, 17.5 mmol D-glucose, 14.3 mmol sodium pyruvate, 15 mmol Hepes, 0.01 mg insulin, 100000 units of penicillin, 100 mg of streptomycin, and 0.250 mg amphotericin B per liter of medium, and devoid of Ile, Leu, Met, and Thr. The medium was adjusted to pH 7.4 and filtered through a 0.22 μm filter. Individual treatment media were prepared by addition of variable amounts of $_{L}$-isoleucine, $_{L}$-leucine, $_{L}$-methionine, and $_{L}$-threonine.

Mammary tissue slices (0.121± 0.016 g) were prepared using a Stadie-Riggs hand held microtome (Thomas Scientific). All slices were weighed, and randomly assigned to 25-ml Erlenmeyer flasks containing 5 ml of treatment media. Flasks were purged for 30 s with 95:5 O_2:CO_2, sealed with a rubber stopper and incubated in a shaking water bath (37ºC and 60 oscillation/min) for 240 min. Preliminary data showed that $^2H_5$ *Phe* enrichment of the pH 4.6 intracelluar protein precipitate increased linearly from 0.5 to 8 h (0.43 ± 0.04 % enrichment increase / h, adjusted $R^2 = 0.93$) indicating tissue viability for the incubation length selected.

Following incubation, slices were washed with 1 mmol/l NaF and 10µmol/l Na_3VO_4 in PBS, homogenized in lysis buffer (7:1, v/m; 50 mmol Tris HCl pH 7.4, 150 mmol NaCl, 1 mmol EDTA, 1 mmol PMSF, 1 mmol Na_3VO_4, 1mmol NaF, 0.001 µg aprotinin, 0.001 µg leupeptin. 0.001 µg pepstatin, 10 ml NP-40, 2.5 g Na-deoxycholate per liter) and centrifuged 5 min at 16000 x *g*. The pellet was discarded.

Protein Immmunoblotting

Aliquots of cell homogenate were combined 5:1 (v/v) with 6X sample buffer (Laemmli, 1970), incubated at 70ºC for 10 min and stored at -80ºC until analysis. Proteins (40 µg) were electrophoretically separated (Laemmli, 1970) and subsequently transferred to Immobilon-FL PVDF membranes (Millipore). Blots were blocked for 1 hr (StartingBlock, Thermo Scientific) and incubated overnight at 4ºC in primary rabbit and mouse antibodies (1:1000 v:v in blocking buffer) against total and site-specific phosphorylated mTOR (Ser2448), eEF2 (Thr56), rpS6 (Ser235/236), and eIF2 (Ser51) (Cell Signaling Technology, except for eEF2 which was purchased from Santa Cruz Biotechnology). After five washes (5 min each) with 0.01 % Tween 20 PBS (Bio-Rad

Life Science), blots were incubated 1 hr at room temperature in goat anti-rabbit and anti-mouse antibodies (1:10000 v:v in blocking buffer; IRDye 800 CW and IRDye 680 respectevely, LI-COR Bioscinces), washed five more times as described and scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences). Signal intensity of total and phosphorylated forms of target proteins was quantified with Odyssey application software (version 3.0) and the phosphorylated to total ratio was calculated.

Intracellular Free AA

Aliquots of cell homogenate were acidified with 50 % (v:v) sulfosalycilic acid (1:4 m:m) and centrifuged 10 min at 10,000 x *g*. Supernatants were gravimetrically combined with ^{13}C universally labeled AA mixture (Cambridge Isotope Laboratories Inc., catalog nos. CLM-1548-1) desalted by ion chromatography (AG 50W-X8 resin, Bio-Rad) and freeze-dried. Amino acids were brought back into solution in acetonitrile (J.T. Baker) and converted to N-(tertbutyldimethyl) AA derivatives by incubation for 1 h at 70ºC in N-Methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (Selectra Sil, UCT). Amino acid derivatives were separated by gas chromatography (Trace GC Ultra, Thermo Scientific) and quantified by mass spectrometry (DSQII, Thermo Scientific) as previously described (Calder et al., 1999). Calibration curves for the labeled AA mixture were generated gravimetrically using an AA standard (AAS18, Sigma-Aldrich).

Experimental Design and Statistical Analysis

The effect of Ile, Leu, Met, and Thr on intracellular AA concentrations and phosphorylation state of signaling proteins was studied with a composite design as described by Park et al. (1976) with modifications, plus one treatment with the base medium and another with all four AA at 100% of DMEM concentrations (Table 3.1). The

composite design consisted of four central points, 2 axial points per AA, and a complete 2^4 factorial. The central points were set at 35% of DMEM concentrations for the four AA studied. This variation from a pure central composite design results in the loss of rotatability (Bohé et al., 2001) and orthogonality of the parameters (St-Pierre and Weiss, 2009), but it was intended to better describe the curvilinear part of the response surface (Appuhamy, 2010). Axial points for each of the 4 AA were set at 0 and 100% of DMEM concentrations, holding the other 3 AA at 35% of DMEM concentrations. Factorial points were set equidistant from central runs at 20 and 50% of DMEM concentrations. The two additional treatments, plus the central points, and factorial treatments with the four AA at 20 and 50% of DMEM concentrations were used to determine the response to the four AA. The experiment was replicated in 5 cows. Data were analyzed with the following statistical model:

$$y_{ijk} = \beta_0 + \sum_{i=1}^{4} \beta_{1_i} x_i + \sum_{i=1}^{4} \sum_{j=i}^{4} \beta_{2_{ij}} x_i x_j + z_k + e_{ijk}$$

$$z_k \sim IIDN(0, \sigma_z^2) \text{ and } e_{ijk} \sim IIDN(0, \sigma_e^2)$$

where y_{ijk} is the response of the i^{th} and j^{th} AA and k^{th} cow. x_i represents the concentration of each of the four EAA in the medium (mmol/l). β_0, β_1, and β_2 are the intercept, linear, and quadratic parameter estimates, respectively; z_k represents the random effect of cow, and e_{ik} the random error. Model parameters, predicted values, and standard errors were estimated with the GLIMMIX procedure of SAS (version 9.3, SAS Institute Inc.). Model selection was performed by backwards elimination removing the least significant quadratic and one-way interactions sequentially until all remaining quadratic terms were significant at $P < 0.1$. Non-significant linear terms that were not associated with any of

the remaining quadratic terms were also removed. Interactions between AA were further tested by calculating, at each level (0, 20, 35, 50, and 100% of DMEM concentrations) of the first AA, the difference between the two levels (20 and 50% of DMEM concentrations) of the second AA of the interaction. Standard errors and confidence limits of the differences were estimated, and compared with the difference at zero concentration. Significance ($P < 0.05$) in the difference between levels of the second AA at different levels of the first AA indicated interaction between them in non-linear responses. Root mean square prediction errors (RMSPE) were calculated as:

$$RMSPE = \sqrt{\frac{\sum {r_i}^2}{n}}$$

where r_i represents the residual for the i^{th} observation and n represent the number of observations. Mean square prediction errors (MSPE) were fractioned into mean bias (MB), and slope bias (SB), which were calculated as:

$$MB = \left(\frac{\sum r_i}{n}\right)^2$$

$$SB = MSPE - MB - \frac{SSE}{n}$$

where SSE represents the error sum of squares of the regression of residuals on predicted values. Both MB and SB were expressed as a percent of the MSPE.

RESULTS AND DISCUSSION

Tissue concentrations of Ile, Leu, Met, and Thr increased linearly in response to supply of those AA in the medium ($P \leq 0.001$; Figure 3.1) confirming the expected effect of treatments. The phosphorylated to total ratio should not be interpreted as the

proportion of total protein that is phosphorylated because signal intensity is affected by antibody affinity to the phosphorylated and total peptides. In fact, ratios larger than one are reported, which would not be possible if it represented the proportion of total protein that is phosphorylated. However, they do represent the relative ratio of phosphorylated to total protein.

Combined Essential Amino Acid Effects

Addition of Ile, Leu, Met, and Thr together had no effect on the ISR pathway as determined by eIF2α (Ser51) phosphorylation (Table 3.2). The ISR pathway is recognized for its specificity to sense AA imbalance rather than AA deficiency (Kimball and Jefferson, 2005). Mouse brain cells detect Leu deficiency and respond by increasing eIF2α phosphorylation and activating an aversive response for the unbalanced diet (Maurin et al., 2005). The liver is one of the first places where an AA unbalance can be detected. Mouse liver cells detect His or Leu deficiency and increase phosphorylation of eIF2α, reducing rate of protein synthesis (Anthony et al., 2004, Zhang et al., 2002). In rats, sulfur AA had similar effects (Sikalidis and Stipanuk, 2010).

Conversely, other studies have reported activation of the eIF2 pathway in response to AA deficiencies rather than imbalance. Rat placenta responded to a balanced low protein diet, activating the ISR pathway through eIF2α phosphorylation (Strakovsky et al., 2010). In mammary tissue slices and MAC-T cells, removal of individual AA from DMEM had no effect on eIF2α phosphorylation, but depletion of all EAA did (Appuhamy et al., 2011a, 2012). In AA-starved MAC-T cells, addition of His, Phe, or Val reduced eIF2α phosphorylation (Arriola Apelo et al., 2010). Infusion, of all EAA plus glucose or His alone, but not Leu nor Met plus Lys to feed-deprived cows reduced

eIF2α phosphorylation. The 4 AA restricted in the present study have not previously been shown to affect the ISR pathway in mammary tissue. Differences in incubation time could have contributed to the differences between this and previous reports in mammary tissue slices, but that is less likely given that maintained signal for up to 6 days has been previously reported in mice (Anthony et al., 2004). Mammary tissue may respond to different AA than liver and brain, or may have lower sensitivity than those tissues for AA. Therefore, more research is needed to understand ISR pathway regulation in peripheral tissues, and specifically mammary glands, as well as its quantitative role in protein synthesis.

The response of mTOR (Ser2448), eEF2 (Thr56), and rpS6 (Ser235/236) to the 4 AA was curvilinear. The effect of EAA on mTOR pathway is widely demonstrated in splanchnic, muscle and mammary tissues (Appuhamy et al., 2011a, Murgas Torrazza et al., 2010, O'Connor et al., 2004). However, these studies were designed to determine a linear response, and cannot identify curvilinear patterns. The present study demonstrates that mTOR responses to Ile, Leu, Met, and Thr are saturable. First derivative analyses showed maximum responses (minimum for eEF2) at 60% of DMEM concentrations for the 4 AA studied, for mTOR and eEF2, and 49% of DMEM concentrations for rpS6. Sixty percent of DMEM concentrations corresponds with 230, 150, 440, and 130% of the plasma concentrations of Ile, Leu, Met, and Thr, respectively, observed in lactating cows fed a 16% CP diet (Appuhamy et al., 2011b). The mTOR responses to the 4 AA at DMEM concentrations did not differ from the maximum response observed (Figure 3.2a), indicating possible saturation within physiological ranges. That type of response justifies our experimental approach with the central point set at 35% of DMEM

concentrations rather than equidistant from the axial points. In the latter case, we would have had too many observations on the plateau and less in the curvilinear region.

The same was not observed for eEF2 and rpS6, where the response at DMEM concentrations differed from the minimum and maximum, respectively. A negative response to these 4 AA in eEF2 (Figure 3.2b) and rpS6 (Figure 3.2c) phosphorylation cannot be explained by enzyme capacity saturation. Rather, they suggest antagonisms between AA, which could also explain the lack of response of eIF2α. It has been shown that excess Thr decreases rate of protein synthesis in skeletal muscle of young pigs (Wang et al., 2007). In murine mammary cells, Thr, Lys, and His, individually decreased rates of protein synthesis, and S6K1 phosphorylation, which phosphorylates rpS6 (Prizant and Barash, 2008). In the present study, Lys and His remained at DMEM concentrations, so, these AA, together with Thr, could have dampened the response to the other 3 AA. Prediction errors were large for eEF2, and especially for rpS6, however, there was no detectable mean or slope bias. A large proportion of dispersion in prediction errors is associated with the assay, and does not necessary implicate lack of model accuracy. Coefficients of variation of 22 and 25% were detected within and between gels for mTOR, respectively (data not shown).

Amino Acid Individual Effects and Interactions

The independent effects of Ile, Leu, Met, and Thr, as well as interactions between those AA, on phosphorylation state of eIF2α (Ser51), mTOR (Ser2448), eEF2 (Thr56), and rpS6 (Ser235/236) were analyzed with a polynomial model, which included linear, quadratic, and one-way interaction terms (Table 3.3).

There were no effects of AA on eIF2α phosphorylation. Only, Ile remained in the model and tended to increase eIF2α phosphorylation ($P = 0.06$). Prediction errors were large (35% of the mean), but there was no mean or slope bias. This response was surprising because phosphorylated eIF2α inhibits translation initiation. Other AA have been shown to have a detrimental effect on protein synthesis in murine mammary cells (Prizant and Barash, 2008), which would correlate with increased eIF2α phosphorylation, but Ile has been shown to stimulate protein synthesis (Appuhamy et al., 2012). Furthermore, as discussed below, Ile significantly activated the mTOR pathway, which is known to stimulate translation. No responses to Leu, Met, and Thr on eIF2α phosphorylation have been previously observed in mammary tissue slices or in vivo experiments (Appuhamy et al., 2012, Toerien et al., 2010).

Contradicting the ISR pathway observations, Ile stimulated the mTOR pathway, suggesting that this is one of the main signaling AA on mRNA translation in the mammary gland (Table 3.3). The mTOR model was reduced to linear effects of Ile ($P = 0.03$) and Thr ($P = 0.04$), and the one-way interaction between these two AA ($P = 0.05$). Prediction errors were markedly lower than for eIF2α phosphorylation (12.6 %), and no MB or SB were detected. Increasing Thr concentration in the medium from 20 to 50% of DMEM concentrations inverted the response observed to Ile (Figure 3.3a). Similarly, when Ile concentration increased, the response to Thr turned from positive to negative (Figure 3.3b). Predictions were done while holding the secondary AA (Thr in Figure 3.3a and Ile in Figure 3.3b) at 20 and 50% of DMEM concentrations because that was the range studied. Predicting the response to either Ile or Thr while holding the other AA at 0 or DMEM concentrations would be extrapolating out of the data range. Leucine and

Met were held at 35% of DMEM concentrations for this analysis. As mentioned above, most of the studies that explored the effect of AA on mTOR and other signaling proteins were not designed to detect curvilinear responses and interactions between AA. Despite this, interactions among AA and energy on phosphorylation of mTOR have been previously identified (Prizant and Barash, 2008, Rius et al., 2010).

Leu is widely recognized as the EAA with largest effect on mTOR phosphorylation and protein synthesis in skeletal muscle, but not in the liver (Murgas Torrazza et al., 2010). It is worth noting that the liver of a lactating cow takes up minimal amounts of branched chain AA. Thus, peripheral tissues and specifically muscle could interpret increased peripheral concentrations of Leu as reflecting higher protein supply and thus a stimulus for increased protein synthesis. In the mammary gland, Leu and Ile appear to share the role of sensor AA (Appuhamy et al., 2012), which seems a logical strategy to diversify the signaling from muscle. A lower sensitivity of the mTOR pathway to Leu in mammary tissue than in muscle could be a potential explanation to the lack of response in the present study. It is also possible that Leu antagonizes with other EAA (e.g. His, Lys) which were held at DMEM concentrations, negating mTOR response to Leu in this study.

The prediction model for eEF2 included linear effects of Ile ($P = 0.02$) and Leu ($P = 0.002$), curvilinear effects of Met (Met, $P = 0.03$; Met × Met, $P = 0.03$) and Thr (Thr, $P = 0.9$; Thr × Thr, $P = 0.03$), and one-way interactions between Leu and Ile ($P = 0.02$), and Thr ($P = 0.07$). Prediction errors for eEF2 phosphorylation were twice those observed for mTOR. However, no increase in mean or slope bias was detected. Phosphorylation of eEF2 by eEF2K, a calmodulin-dependent kinase downstream of mTOR and S6K1, inhibits its translation elongation activity. Linear and quadratic parameters were

negative, thus, indicating positive effects of AA on translation elongation. In agreement with what was previously observed for mTOR, Ile linearly decreased eEF2 phosphorylation (Figure 3.4a). Leucine also decreased eEF2 phosphorylation (Figure 3.4b). Eukaryotic elongation factor 2 is downstream of mTOR in the pathway, so, Leu effect on eEF2 but not on mTOR, supports the hypothesis of lower Leu affinity in the mammary gland than in muscle. As the signals pass from one protein to another, it gets magnified because one phosphorylated mTOR molecule can phosphorylate many downstream substrates. Furthermore, in this case eEF2 is three steps downstream in the pathway, so that the magnification effect can get potentiated several fold. Effects of Met on eEF2 phosphorylation had not been reported in mammary tissue. However, despite linear and quadratic terms being included in the model, no treatment level was different than any other based on confidence limits (Figure 3.4c). Threonine effects on eEF2 phosphorylation were evident only at DMEM concentration, and when Leu was held at 20%, but not at 50% of DMEM concentrations (Figure 3.4d). This suggests a milder effect (lower sensitivity) of Thr, than Ile and Leu, on eEF2 phosphorylation. Previous studies predicted a linear response of eEF2 to EAA supply (Appuhamy et al., 2011a), but not to Ile or Leu alone (Appuhamy et al., 2012), possibly because that study used a deletion approach, so antagonistic AA remained high at DMEM concentrations when Ile and Leu responses were analyzed. In Chinese hamster ovary cells expressing human insulin receptors, insulin inhibited eEF2 phosphorylation in a rapamycin dependent manner, confirming a mTOR pathway dependent mechanism (Redpath et al., 1996). In mammary tissue slices, insulin had no effect on eEF2 phosphorylation (Appuhamy et al., 2011a), although it is unclear whether adequate insulin reached internal portions of the

slices to elicit an effect. Growth hormone affected total eEF2 abundance in lactating cows, but had no effect on phosphorylation state of the protein (Hayashi et al., 2009).

The eEF2 model predicted responses to Ile, Leu and Thr, only when the antagonistic AA (Leu, Ile and Thr, and Leu, respectively) remained at low concentrations. Increasing the concentration of the antagonistic AA to 50% completely negated the response to any of the three AA. Moreover, for Ile, the slope changed (100% of DMEM concentrations was significantly higher than 0% of DMEM concentration) when Leu was increased from 20 to 50% of DMEM concentration. An antagonism between Leu and Thr on mTOR phosphorylation has been previously reported (Prizant and Barash, 2008). Leu and Ile are both transported into the cell by the L transport system (Bequette et al., 2000). Increased concentrations of one could have a negative impact on the transport of the other one, reducing its intracellular concentration and mitigating its effect on eEF2 phosphorylation. Considering only axial points, both AA numerically reduced the concentration of the other one, but more observations are needed to demonstrate a true effect on intracellular concentration. However, both AA had linear effects on eEF2 phosphorylation, so if the medium concentration of one tends to saturate the transporter capacity and inhibit the transport of the second one, at least the first one should maintain its effect on eEF2 phosphorylation, but this was not observed. The question of where the antagonism between AA (in the transport or intracelularly) happens remains unanswered.

Similar to mTOR, the rpS6 model included linear effect of Ile ($P = 0.003$), quadratic effect of Thr (Thr^2, $P = 0.07$), and a negative interaction between the two ($P = 0.007$). Prediction errors were the largest for this protein. However, as for the other three proteins, mean and slope biases were irrelevant, and most of the error was attributed to

dispersion. Ile linearly increased rpS6 phosphorylation (Figure 3.5a) when Thr concentrations were held at 20% of DMEM. However, increasing Thr concentrations to 50% of DMEM negated that effect. Thr increased rpS6 phosphorylation curvilinearly when Ile was held at 20% of DMEM concentration, with most of the effect observed between 50% and DMEM concentrations (Figure 3.5b). However, increasing Ile concentrations to 50% of DMEM inverted Thr effects on rpS6 phosphorylation (0 > 100% of DMEM concentration). Ile increased rpS6 phosphorylation in MAC-T cells, but not in mammary tissue slices (Appuhamy et al., 2012). As mentioned above, Thr and other EAA remained high (DMEM concentrations) in that study, possibly negating the effect of Ile. In vivo, venous infusion of all EAA or Leu alone increased rpS6 phosphorylation in the mammary glands (Toerien et al., 2010). Considering AA antagonisms, addition studies like that of Toerien et al. (2010) may be better suited to unveil individual AA effects. However, even those studies did not reveal antagonisms observed in the present study, nor did they allow increased understanding of the curvilinear response to increased supply of specific AA.

Despite differences in the prediction model, mTOR and rpS6 responded to Ile and Thr, and this was reflected in the positive correlation between the two proteins (Table 3.4; $P = 0.02$). This is expected given that the former protein is upstream in the pathway. In addition, mTOR was negatively correlated with eEF2 ($P < 0.001$). That is also expected because eEF2 phosphorylation is inhibited by the mTOR pathway downstream kinase S6K1, and because eEF2 phosphorylation decreases elongation rate, and mTOR has a positive effect on CFSR. Ribosomal protein S6 was negatively correlated with eEF2 ($P < 0.001$), demonstrating significant agreement between branches within the pathway.

Moreover, mTOR and eIF2α were negatively correlated ($P = 0.001$), indicating no redundancies between pathways, given that they have opposite effects on protein synthesis.

CONCLUSIONS

This study confirms previous reports indicating that the mTOR pathway was the most sensitive of the two pathways to AA signaling. It also confirmed that, contrary to liver and skeletal muscle, in the mammary glands Ile and Leu are the strongest signaling AA for translation regulation. In addition, it was demonstrated that the protein signaling response to these AA is conditioned by antagonisms between these and with other AA. These findings will help to improve mathematical models of casein translation regulation in the mammary glands.

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REFERENCES

Anthony, T. G., B. J. McDaniel, R. L. Byerley, B. C. McGrath, D. R. Cavener, M. A. McNurlan, and R. C. Wek. 2004. Preservation of liver protein synthesis during dietary leucine deprivation occurs at the expense of skeletal muscle mass in mice deleted for eif2 kinase gcn2. J. Biol. Chem. 279(35):36553-36561.

Appuhamy, J. A. D. R. N. 2010. Regulatory roles of essential amino acids, energy, and insulin in mammary cell protein synthesis. in Animal Science, Dairy. Vol. Doctor of Philosophy. Virginia Polytechnic Institute and State University, Blacksburg, VA.

Appuhamy, J. A. D. R. N., A. L. Bell, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2011a. Essential amino acids regulate both initiation and elongation of mrna translation independent of insulin in mac-t cells and bovine mammary tissue slices. J. Nutr. 141(6):1209-1215.

Appuhamy, J. A. D. R. N., J. R. Knapp, O. Becvar, J. Escobar, and M. D. Hanigan. 2011b. Effects of jugular-infused lysine, methionine, and branched-chain amino acids on milk protein synthesis in high-producing dairy cows. J. Dairy Sci. 94(4):1952-1960.

Appuhamy, J. A. D. R. N., N. A. Knoebel, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2012. Isoleucine and leucine independently regulate mtor signaling and protein synthesis in mac-t cells and bovine mammary tissue slices. J. Nutr. 142(3):484-491.

Arriola Apelo, S. I., W. A. D. Nayananjalie, J. A. D. R. N. Appuhamy, and M. D. Hanigan. 2010. M-tor independent model of protein synthesis regulation by essential amino acids in mammary epithelial cells. Page 247 in Energy and protein metabolism and nutrition. M. G. Crovetto, ed. Wageningen Academic, Parma, Italy.

Bequette, B. J., M. D. Hanigan, A. G. Calder, C. K. Reynolds, G. E. Lobley, and J. C. MacRae. 2000. Amino acid exchange by the mammary gland of lactating goats when histidine limits milk production. J. Dairy Sci. 83(4):765-775.

Bohé, J., J. F. A. Low, R. R. Wolfe, and M. J. Rennie. 2001. Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. The Journal of Physiology 532(2):575-579.

Calder, A. G., K. E. Garden, S. E. Anderson, and G. E. Lobley. 1999. Quantitation of blood and plasma amino acids using isotope dilution electron impact gas chromatography/mass spectrometry with u-13c amino acids as internal standards. Rapid Commun. Mass Spectrom. 13(21):2080-2083.

Caraviello, D. Z., K. A. Weigel, P. M. Fricke, M. C. Wiltbank, M. J. Florent, N. B. Cook, K. V. Nordlund, N. R. Zwald, and C. L. Rawson. 2006. Survey of management practices on reproductive performance of dairy cattle on large us commercial farms. J. Dairy Sci. 89(12):4723-4735.

Davis, T. A., M. L. Fiorotto, D. G. Burrin, P. J. Reeds, H. V. Nguyen, P. R. Beckett, R. C. Vann, and P. M. J. O'Connor. 2002. Stimulation of protein synthesis by both insulin and amino acids is unique to skeletal muscle in neonatal pigs. Am. J. Physiol. Endocrinol. Metab. 282(4):E880-890.

Dunlop, E. A. and A. R. Tee. 2009. Mammalian target of rapamycin complex 1: Signalling inputs, substrates and feedback mechanisms. Cell. Signal. 21(6):827-835.
Escobar, J., J. W. Frank, A. Suryawan, H. V. Nguyen, S. R. Kimball, L. S. Jefferson, and T. A. Davis. 2006. Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs. Am. J. Physiol. Endocrinol. 290(4):E612-E621.
Hanigan, M. D., J. P. Cant, D. C. Weakley, and J. L. Beckett. 1998. An evaluation of postabsorptive protein and amino acid metabolism in the lactating dairy cow. J. Dairy Sci. 81(12):3385-3401.
Hanigan, M. D., J. France, L. A. Crompton, and B. J. Bequette. 2000. Evaluation of a representation of the limiting amino acid theory for milk protein synthesis. in Modelling nutrient utilization in farm animals. J. P. McNamara, J. France, and D. E. Beever, ed. CABI, Wallingford.
Hanigan, M. D., C. K. Reynolds, D. J. Humphries, B. Lupoli, and J. D. Sutton. 2004. A model of net amino acid absorption and utilization by the portal-drained viscera of the lactating dairy cow. J. Dairy Sci. 87(12):4247-4268.
Haque, M. N., H. Rulquin, A. Andrade, P. Faverdin, J. L. Peyraud, and S. Lemosquet. 2012. Milk protein synthesis in response to the provision of an “ideal” amino acid profile at 2 levels of metabolizable protein supply in dairy cows. J. Dairy Sci. 95(10):5876-5887.
Hayashi, A. A., K. Nones, N. C. Roy, W. C. McNabb, D. S. Mackenzie, D. Pacheco, and S. McCoard. 2009. Initiation and elongation steps of mrna translation are involved in the increase in milk protein yield caused by growth hormone administration during lactation. J. Dairy Sci. 92(5):1889-1899.
Kimball, S. R. 2002. Regulation of global and specific mrna translation by amino acids. J. Nutr. 132(5):883-886.
Kimball, S. R. and L. S. Jefferson. 2005. Role of amino acids in the translational control of protein synthesis in mammals. Semin. Cell Dev. Biol. 16(1):21-27.
Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage t4. Nature 227(5259):680-685.
Lansard, M., S. p. Panserat, E. Plagnes,ÄêJuan, K. Dias, I. Seiliez, and S. Skiba,ÄêCassy. 2011. L-leucine, l-methionine, and l-lysine are involved in the regulation of intermediary metabolism-related gene expression in rainbow trout hepatocytes. J. Nutr. 141(1):75-80.
Lapierre, H., C. E. Galindo, S. Lemosquet, I. Ortigues-Marty, L. Doepel, and D. R. Ouellet. 2010. Protein supply, glucose kinetics and milk yield in dairy cows. Pages 275-286 in Energy and protein metabolism and nutrition. G. M. Crovetto, ed. Wageningen Academic Publishers, Wageningen.
MacRae, J. C., L. A. Bruce, D. S. Brown, and A. G. Calder. 1997. Amino acid use by the gastrointestinal tract of sheep given lucerne forage. American Journal of Physiology - Gastrointestinal and Liver Physiology 273(6):G1200-G1207.
Mahoney, S. J., J. M. Dempsey, and J. Blenis. 2009. Chapter 2 cell signaling in protein synthesis: Ribosome biogenesis and translation initiation and elongation. Pages 53-107 in Progress in molecular biology and translational science. Vol. Volume 90. W. B. H. John, ed. Academic Press.

Maurin, A.-C., C. Jousse, J. Averous, L. Parry, A. Bruhat, Y. Cherasse, H. Zeng, Y. Zhang, H. P. Harding, D. Ron, and P. Fafournoux. 2005. The gcn2 kinase biases feeding behavior to maintain amino acid homeostasis in omnivores. Cell Metab. 1(4):273-277.

Metayer-Coustard, S., H. Mameri, I. Seiliez, S. Crochet, P. Crepieux, Y. Mercier, P.-A. Geraert, and S. Tesseraud. 2010. Methionine deprivation regulates the s6k1 pathway and protein synthesis in avian qm7 myoblasts without activating the gcn2/eif2 alpha cascade. J. Nutr. 140(9):1539-1545.

Moshel, Y., R. E. Rhoads, and I. Barash. 2006. Role of amino acids in translational mechanisms governing milk protein synthesis in murine and ruminant mammary epithelial cells. J. Cell. Biochem. 98(3):685-700.

Murgas Torrazza, R., A. Suryawan, M. C. Gazzaneo, R. n. A. Orellana, J. W. Frank, H. V. Nguyen, M. L. Fiorotto, S. El-Kadi, and T. A. Davis. 2010. Leucine supplementation of a low-protein meal increases skeletal muscle and visceral tissue protein synthesis in neonatal pigs by stimulating mtor-dependent translation initiation. J. Nutr.

O'Connor, P. M. J., S. R. Kimball, A. Suryawan, J. A. Bush, H. V. Nguyen, L. S. Jefferson, and T. A. Davis. 2004. Regulation of neonatal liver protein synthesis by insulin and amino acids in pigs. Am. J. Physiol. Endocrinol. 286(6):E994-E1003.

Park, C. S., P. T. Chandler, and R. M. Clark. 1976. Optimum amino acid complement for protein synthesis by rat mammary cells in tissue culture1. J. Dairy Sci. 59(10):1758-1763.

Prizant, R. L. and I. Barash. 2008. Negative effects of the amino acids lys, his, and thr on s6k1 phosphorylation in mammary epithelial cells. J. Cell. Biochem. 105(4):1038-1047.

Raggio, G., D. Pacheco, R. Berthiaume, G. E. Lobley, D. Pellerin, G. Allard, P. Dubreuil, and H. Lapierre. 2004. Effect of level of metabolizable protein on splanchnic flux of amino acids in lactating dairy cows. J. Dairy Sci. 87(10):3461-3472.

Redpath, N. T., E. J. Foulstone, and C. G. Proud. 1996. Regulation of translation elongation factor-2 by insulin via a rapamycin-sensitive signalling pathway. EMBO J. 15(9):2291-2297.

Rius, A. G., J. A. D. R. N. Appuhamy, J. Cyriac, D. Kirovski, O. Becvar, J. Escobar, M. L. McGilliard, B. J. Bequette, R. M. Akers, and M. D. Hanigan. 2010. Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. J. Dairy Sci. 93(7):3114-3127.

Sikalidis, A. K. and M. H. Stipanuk. 2010. Growing rats respond to a sulfur amino acid-deficient diet by phosphorylation of the {alpha} subunit of eukaryotic initiation factor 2 heterotrimeric complex and induction of adaptive components of the integrated stress response. J. Nutr. 140(6):1080-1085.

St-Pierre, N. R. and W. P. Weiss. 2009. Technical note: Designing and analyzing quantitative factorial experiments1. J. Dairy Sci. 92(9):4581-4588.

Strakovsky, R. S., D. Zhou, and Y. Ä. Pan. 2010. A low-protein diet during gestation in rats activates the placental mammalian amino acid response pathway and programs the growth capacity of offspring. J. Nutr.

Toerien, C. A., D. R. Trout, and J. P. Cant. 2010. Nutritional stimulation of milk protein yield of cows is associated with changes in phosphorylation of mammary eukaryotic initiation factor 2 and ribosomal s6 kinase 1. J. Nutr. 140(2):285-292.

Wang, X., S. Qiao, Y. Yin, L. Yue, Z. Wang, and G. Wu. 2007. A deficiency or excess of dietary threonine reduces protein synthesis in jejunum and skeletal muscle of young pigs. J. Nutr. 137(6):1442-1446.

Whitelaw, F. G., J. S. Milne, E. R. Ørskov, and J. S. Smith. 1986. The nitrogen and energy metabolism of lactating cows given abomasal infusions of casein. Br. J. Nutr. 55(03):537-556.

Wolfe, A. H. and J. A. Patz. 2002. Reactive nitrogen and human health:Acute and long-term implications. Ambio 31(2):120-125.

Zhang, P., B. C. McGrath, J. Reinert, D. S. Olsen, L. Lei, S. Gill, S. A. Wek, K. M. Vattem, R. C. Wek, S. R. Kimball, L. S. Jefferson, and D. R. Cavener. 2002. The gcn2 eif2{alpha} kinase is required for adaptation to amino acid deprivation in mice. Mol. Cell. Biol. 22(19):6681-6688.

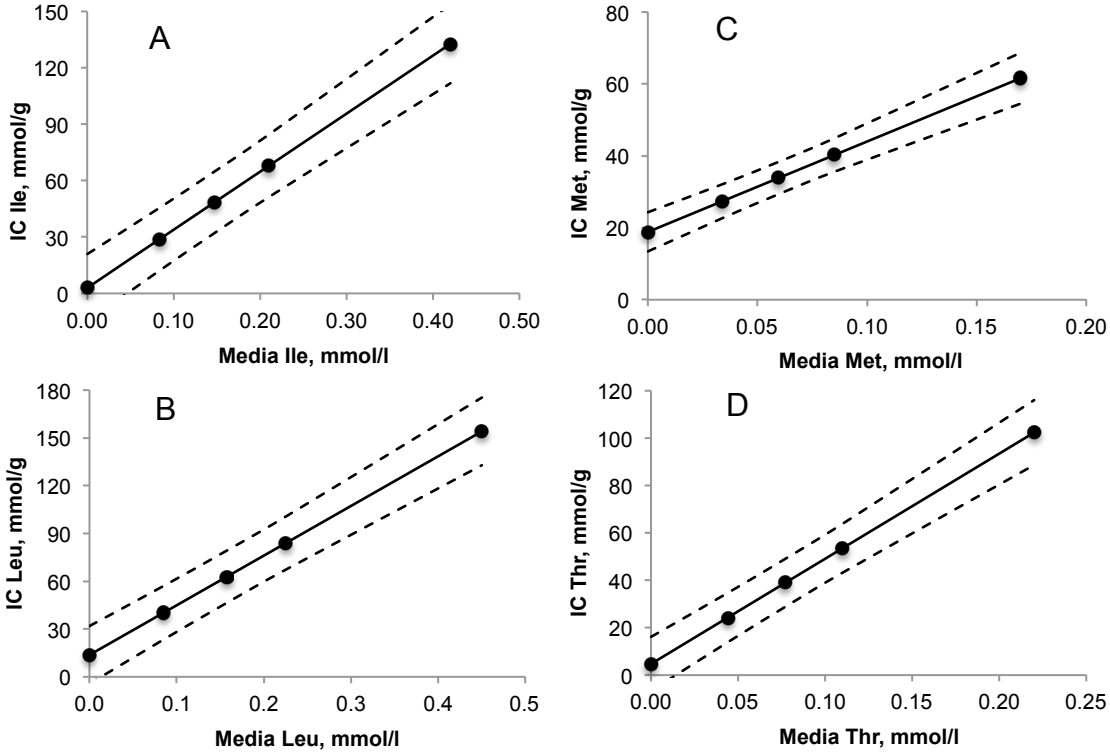

A
IC Ile, mmol/g
Media Ile, mmol/l
B
IC Leu, mmol/g
Media Leu, mmol/l
C
IC Met, mmol/g
Media Met, mmol/l
D
IC Thr, mmol/g
Media Thr, mmol/l

Figure 3.1. Intracellular concentration (mmol/g wet tissue) of Ile (A), Leu (B), Met (C), and Thr (D) in response to media concentrations (mmol/l) of the respective AA. The solid line represents predicted concentrations. Markers represent least square estimates of AA concentrations at 0, 20, 35, 50, and 100% of DMEM concentrations. Dashed lines represent 95% confident limits. IC: intracellular.

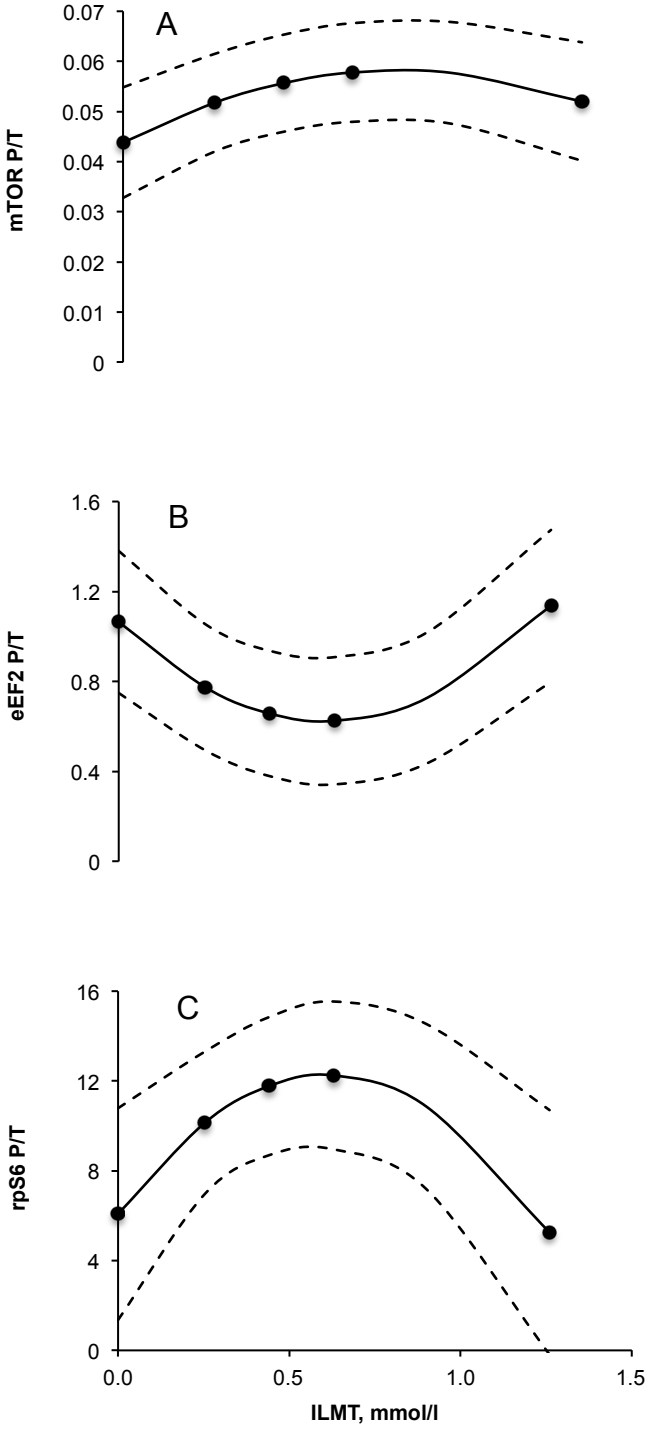
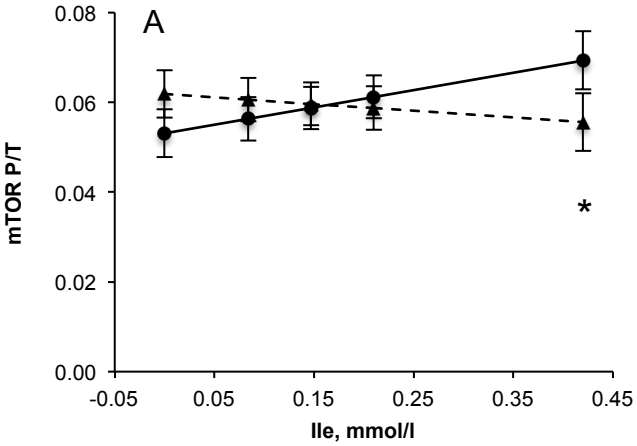

A
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
mTOR P/T
B
1.6
1.2
0.8
0.4
0
eEF2 P/T
C
16
12
8
4
0
rpS6 P/T
0.0
0.5
1.0
1.5
ILMT, mmol/l

Figure 3.2. Ile, Leu, Met, and Thr (ILMT, mmol/l) effect on phosphorylated to total ratios (P/T) of mammalian target of rapamycin (mTOR Ser2448; panel A), eukaryotic elongation factor 2 (eEF2 Thr56; panel B), and ribosomal protein S6 (rpS6 Ser235/236). The solid line represents predicted concentrations. Markers represent least square estimates of AA concentrations at 0, 20, 35, 50, and 100% of DMEM concentrations. Dashed lines represent 95% confident limits.


A
0.08
0.06
0.04
0.02
0.00
mTOR P/T
-0.05
0.05
0.15
0.25
0.35
0.45
Ile, mmol/l
*

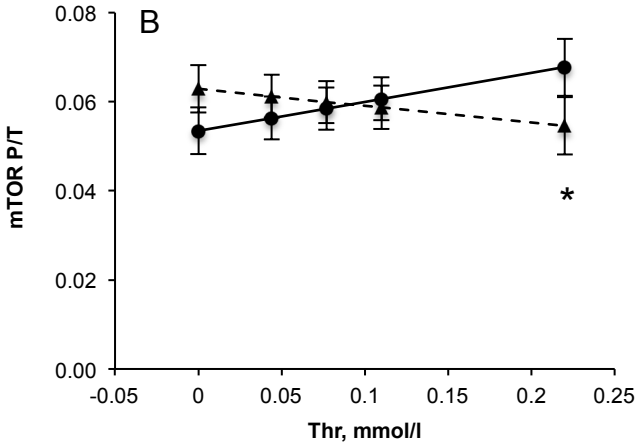
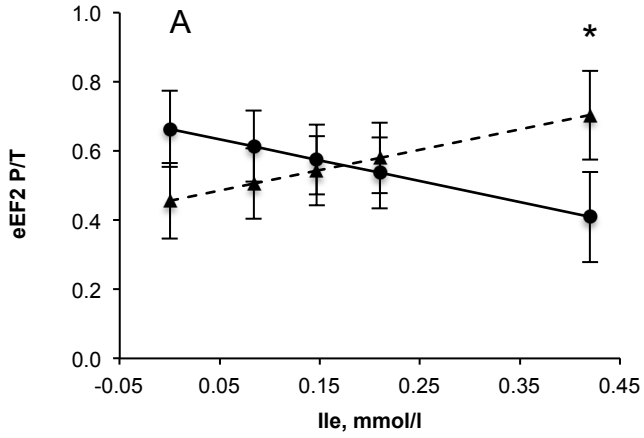
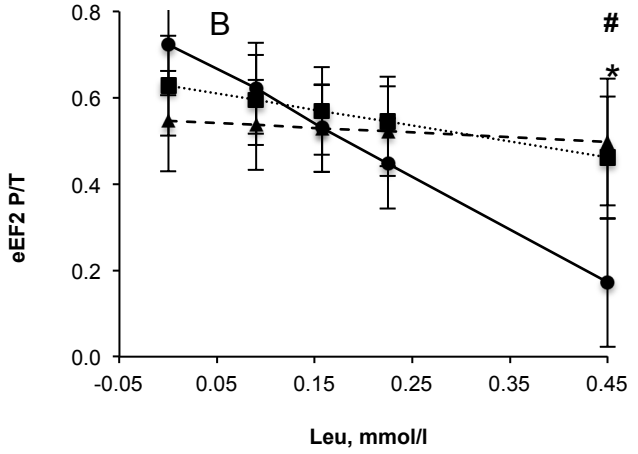
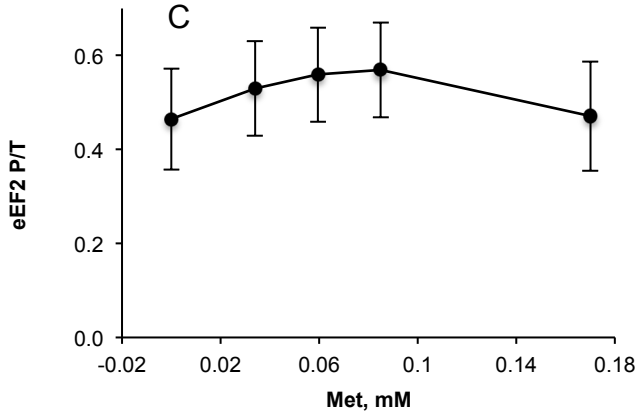

B
0.08
0.06
0.04
0.02
0.00
mTOR P/T
-0.05
0
0.05
0.1
0.15
0.2
0.25
Thr, mmol/l
*

Figure 3.3. Ile (panel A) and Thr (panel B) effect on mTOR (Ser2448) phosphorylated to total ratio (P/T) with Thr (A) or Ile (B) at 20% (solid line with circles), and 50% (dashed line with triangles) of DMEM concentrations.
Markers represent least square estimates of Ile (A) or Thr (B) concentrations at 0, 20, 35, 50, and 100% of DMEM. Stars indicate significance (95% confidence limit) from 0% Ile (A) or Thr (B) for the difference between the two levels of Thr (A) or Ile (B). Leu and Met were held at 35% of DMEM concentrations.


A
*
eEF2 P/T
1.0
0.8
0.6
0.4
0.2
0.0
-0.05
0.05
0.15
0.25
0.35
0.45
Ile, mmol/l


B
#
*
eEF2 P/T
0.8
0.6
0.4
0.2
0.0
-0.05
0.05
0.15
0.25
0.35
0.45
Leu, mmol/l


C
eEF2 P/T
0.6
0.4
0.2
0.0
-0.02
0.02
0.06
0.1
0.14
0.18
Met, mM

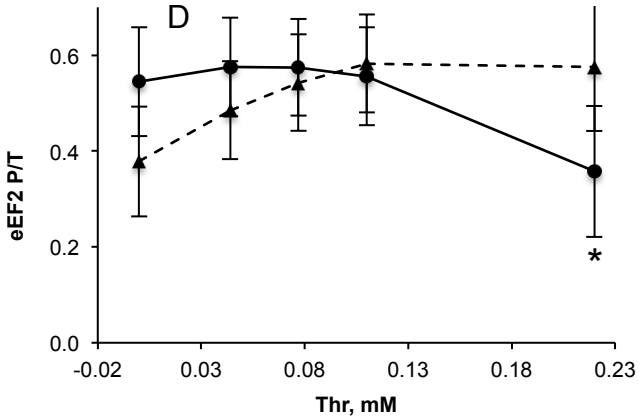

D
*
eEF2 P/T
0.6
0.4
0.2
0.0
-0.02
0.03
0.08
0.13
0.18
0.23
Thr, mM

Figure 3.4. Ile (panel A), Leu (panel B), Met (panel C), and Thr (panel D) effects on eEF2 (Thr56) phosphorylated to total ratios (P/T) with Leu (A and D), Ile and Thr (B) at 20% (solid line with circles), Leu (A and D), Ile (B) 50% of DMEM concentrations (dashed line with triangles), and Thr (B) at 50% of DMEM concentrations (dotted line with squares).
Markers represent least square estimates of Ile (A), Leu (B), Met (C) or Thr (D) at 0, 20, 35, 50, and 100% of DMEM concentrations. Stars indicate significance (95% confidence limits) from 0% Ile (A), Leu (B), or Thr (D) for the difference between the two levels of Leu (A and C), Ile (B), or Thr (B, numeral sign).

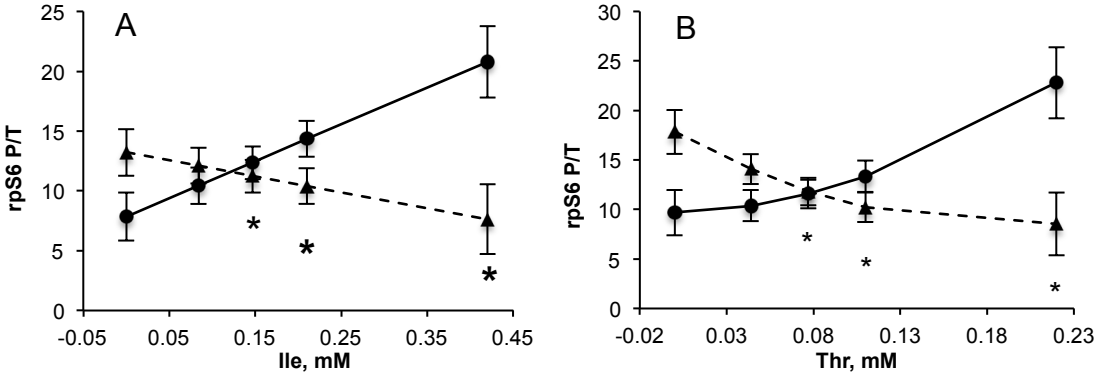

A
rpS6 P/T
Ile, mM
B
rpS6 P/T
Thr, mM

Figure 3.5. Ile (panel A) and Thr (panel B) effects on rpS6 (Ser235/236) phosphorylated to total ratios (P/T) with Thr (A) or Ile (B) at 20% (solid line with circles), and 50% of DMEM concentrations (dashed line with triangles).
Markers represent least square estimates of Ile (A) or Thr (B) at 0, 20, 35, 50, and 100% of DMEM concentrations. Stars indicate significance (95% confidence limits) from 0% Ile (A) or Thr (B) for the difference between the two levels of Thr (A) or Ile (B). Leu and Met were hold at 35% of DMEM concentrations.

Table 3.1. Four factors composite design expressed as code, percent of Dulbecco's Modified Eagle Medium (DMEM) concentrations, and media concentration (mmol/l)

CD[1]				% DMEM				mmol/l			
I[2]	L	M	T	I	L	M	T	I	L	M	T
0	0	0	0	35	35	35	35	0.147	0.158	0.060	0.077
0	0	0	0	35	35	35	35	0.147	0.158	0.060	0.077
0	0	0	0	35	35	35	35	0.147	0.158	0.060	0.077
0	0	0	0	35	35	35	35	0.147	0.158	0.060	0.077
-2.3	0	0	0	0	35	35	35	0.000	0.158	0.060	0.077
4.3	0	0	0	100	35	35	35	0.420	0.158	0.060	0.077
0	-2.3	0	0	35	0	35	35	0.147	0.000	0.060	0.077
0	4.3	0	0	35	100	35	35	0.147	0.450	0.060	0.077
0	0	-2.3	0	35	35	0	35	0.147	0.158	0.000	0.077
0	0	4.3	0	35	35	100	35	0.147	0.158	0.170	0.077
0	0	0	-2.3	35	35	35	0	0.147	0.158	0.060	0.000
0	0	0	4.3	35	35	35	100	0.147	0.158	0.060	0.220
-1	-1	-1	-1	20	20	20	20	0.084	0.090	0.034	0.044
-1	-1	-1	1	20	20	20	50	0.084	0.090	0.034	0.110
-1	-1	1	-1	20	20	50	20	0.084	0.090	0.085	0.044
-1	-1	1	1	20	20	50	50	0.084	0.090	0.085	0.110
-1	1	-1	-1	20	50	20	20	0.084	0.225	0.034	0.044
-1	1	-1	1	20	50	20	50	0.084	0.225	0.034	0.110
-1	1	1	-1	20	50	50	20	0.084	0.225	0.085	0.044
-1	1	1	1	20	50	50	50	0.084	0.225	0.085	0.110
1	-1	-1	-1	50	20	20	20	0.210	0.090	0.034	0.044
1	-1	-1	1	50	20	20	50	0.210	0.090	0.034	0.110
1	-1	1	-1	50	20	50	20	0.210	0.090	0.085	0.044
1	-1	1	1	50	20	50	50	0.210	0.090	0.085	0.110
1	1	-1	-1	50	50	20	20	0.210	0.225	0.034	0.044
1	1	-1	1	50	50	20	50	0.210	0.225	0.034	0.110
1	1	1	-1	50	50	50	20	0.210	0.225	0.085	0.044
1	1	1	1	50	50	50	50	0.210	0.225	0.085	0.110
-2.3	-2.3	-2.3	-2.3	0	0	0	0	0.000	0.000	0.000	0.000
4.3	4.3	4.3	4.3	100	100	100	100	0.420	0.450	0.170	0.220

[1]Composite design
[2]I:Isoleucine, L:Leucine, M:Metionine, T:Threonine

Table 3.2. Parameter estimates and significance level for the combined effect of Ile, Leu, Met, and Thr on signaling proteins.

	eIF2[1] α Ser51 P/T			mTOR[2] Ser2448 P/T			eEF2[3] Thr56 P/T			rpS6[4] Ser235/236		
Effect	Estimate	SE	*P*	Estimate	SE	*P*	Estimate	SE	*P*	Estimate	SE	*P*
Intercept	0.038	0.004	0.001	0.044	0.005	0.001	1.06	0.15	0.002	6.1	2.3	0.058
ILMT[5] (l/mmol)	-0.004	0.006	0.5	0.038	0.010	0.001	-1.45	0.27	<0.001	20.3	6.5	0.004
ILMT × ILMT (l^2/$mmol^2$)				-0.025	0.007	0.001	1.20	0.20	<0.001	-16.6	4.7	0.002
RMSE % mean[6]				10.3			20.4			35.9		
MB % MSE[7]				0.0			0.0			0.0		
SB % MSE[8]				0.5			0.4			1.2		

[1]eIF2: eukaryotic initiation factor 2 α subunit
[2]mTOR: mammalian target of rapamycin
[3]eEF2: eukaryotic elongation factor 2
[4]rpS6: ribosomal protein S6
[5]ILMT: effect of Ile, Leu, Met, and Thr together. Included only treatments with all 4 AA at 0, 20, 35, 50, and 100% of Dulbecco's Modified Eagle Medium concentrations
[6]RMSPE % mean: root mean square prediction error expressed as percent of the sample mean
[7]MB % MSE: mean bias expressed as percent of the mean square prediction error
[8]SB % MSE: slope bias expressed as percent of the mean square prediction error

Table 3.3. Model fitting for Ile, Leu, Met, and Thr effects on signaling proteins

	eIF2α[1] Ser51			mTOR[2] Ser2448			eEF2[3] Thr56			rpS6[4] Ser235/236		
Effect	Estimate	SE	*P*	Estimate	SE	*P*	Estimate	SE	*P*	Estimate	SE	*P*
Intercept	0.032	0.003	0.001	0.047	0.007	<0.001	0.769	0.174	0.012	5.37	3.46	0.2
l/mmol												
ILE	0.028	0.015	0.057	0.074	0.033	0.026	-1.405	0.614	0.024	60.26	19.69	0.003
LEU							-2.540	0.812	0.002			
MET							2.400	1.107	0.032			
THR				0.133	0.063	0.035	-0.115	1.384	0.9	46.55	44.64	0.3
$l^2/mmol^2$												
ILE × ILE												
LEU × LEU												
MET × MET							-13.906	6.464	0.033			
THR × THR							-8.661	3.805	0.025	224.78	120.75	0.065
ILE × LEU							8.864	3.722	0.019			
ILE × MET												
ILE × THR				-0.813	0.410	0.049				-668.75	243.41	0.007
LEU × MET												
LEU × THR							12.964	7.094	0.070			
MET × THR												
RMSE % mean[5]	34.6			12.6			25.2			36.7		
MB % MSE[6]	0.0			0.0			0.0			0.0		
SB % MSE[7]	0.5			0.1			0.0			0.2		

1eIF2α: eukaryotic initiation factor 2 α subunit
2mTOR: mammalian target of rapamycin
3eEF2: eukaryotic elongation factor 2
4rpS6: ribosomal protein S6
5RMSPE % mean: root mean square prediction error expressed as percent of the observed mean
6MB % MSE: mean bias expressed as percent of the mean square prediction error
7SB % MSE: slope bias expressed as percent of the mean square prediction error

Table 3.4. Pearson correlations and *P* values between signaling proteins phosphorylated to total ratios.

		mTOR[2]	eEF2[3]	rpS6[4]
eIF2α[1]	Corr.[5]	-0.28	0.03	0.08
	P	0.001	0.7	0.4
mTOR	Corr.		-0.51	0.12
	P		<0.001	0.020
eEF2	Corr.			-0.37
	P			<0.001

[1]eIF2α: eukaryotic initiation factor 2 α subunit
[2]mTOR: mammalian target of rapamycin.
[3]eEF2: eukaryotic elongation factor 2
[4]rpS6: ribosomal protein S6
[5] Corr.: Pearson correlation

CHAPTER 4: Essential amino acids effects on fractional synthesis rate of casein are saturable and additive.

ABSTRACT

Specific AA affect rates of milk protein synthesis in the mammary glands of lactating cows. The objective of this study was to quantify the response in α-S1 casein synthesis rate to Ile, Leu, Met, and Thr supplementation, and to test the single-limiting AA theory for milk protein synthesis by exploring interactions between these AA. Effects of AA were studied with a composite design containing a central point repeated 4 times, 2 axial points per AA and a complete 2^4 factorial. Other AA were provided at Dulbecco's Modified Eagle Medium (DMEM) concentrations. The experiment was replicated with mammary tissue from 5 lactating cows. Mammary tissue slices (0.12 ± 0.02 g) were incubated for 4 h at 37 ºC in 5 ml of treatment media containing $^2H_5$-Phe. Caseins were precipitated from cell homogenate supernatants. $^2H_5$-Phe enrichment of the N[34]LLRFFVAPFPE α-S1 peptide was determined by MALDI-TOF, and used to determine enrichment of Phe in the tRNA pool and α-S1 casein fractional synthesis rates (CFSR). Data were analyzed with a polynomial mixed model containing linear, quadratic, and one-way interactions for Ile, Leu, Met, and Thr, and cow and residual as random factors. One-way interactions were not significant ($P > 0.1$), and thus they were removed from the model. Increasing concentrations of Ile, Leu, Met, and Thr simultaneously increased CFSR curvilinearly with an estimated maximum response at 63% DMEM and a predicted response of 4.32 ± 0.84 %/h. Prediction errors were large (31.8 %), but they did not exhibit mean or slope bias. The maximum response to each of

the 4 AA was at 71, 49, 60, and 65% of the concentration in DMEM, for Ile, Leu, Met, and Thr, respectively. These values correspond to 270, 120, 440, and 140% the plasma concentrations of Ile, Leu, Met, and Thr observed in a lactating cow fed to meet NRC requirements, respectively. The CFSR estimated at those maximums were similar between AA (3.6 ± 0.6 %/h). Individual AA effects on CFSR did not correlate with mTOR signaling. Independent responses of CFSR to individual essential AA observed in this study contradict the single-limiting AA theory assumed in current requirement systems. The saturable responses in CFSR to these 4 AA also highlight the inadequacy of using a fixed post-absorptive AA efficiency approach for milk protein synthesis.

Keywords: essential amino acid, casein, protein synthesis, mammary gland.

INTRODUCTION

The 2010 revision of the United Nations World Population Prospect estimates the world population will increase approximately 30 % by 2050 (http://esa.un.org/unpd/wpp/index.htm). Feeding the additional people will be challenging given the limited amount of natural resources, specifically land. Therefore, improvements in agricultural technology that increase the production level per unit of land are urgently required. However, production efficiency cannot come at expense of environmental sustainability, as demanded by governments and public opinion.

Ruminants are especially efficient in converting low quality feed into high quality product (i.e. milk, meat, and wool). This is explained by their synergism with microbes that allow them to break ß 1-6 glucose bonds from cellulose, and to incorporate ammonium N into carbon skeletons to synthesize AA that mammalian cells cannot. However, after duodenal absorption, utilization of microbial and dietary AA is significantly less efficient than in swine and poultry. Dairy cattle only capture 25% of dietary N into milk protein, with the remaining excreted to the environment, mainly as urea. Nitrogen export to the environment can result in eutrophication of aquatic ecosystems, increased atmospheric particulates; decreased stratospheric ozone concentration; global warming; increased acidity of soil, precipitation, and surface water; coastal hypoxia; and methemoglobinemia in infants (Wolfe and Patz, 2002).

Swine and poultry industries formulate rations with low protein levels, and supplement with individual AA to meet specific AA requirements, reaching post-absorptive AA efficiencies of 50% or higher. Baker (1996) demonstrated that post-absorptive use of N in pigs can be up to 85% when supply of AA matches tissue needs. In theory, dairy cattle

should not be much different. However, individual AA requirement have been determined for Lys and Met only, and as a proportion of total post-absorptive AA supply (NRC, 2001) . Thus, if total AA requirements are not correct, neither are the requirements for those AA. The NRC (2001) evaluation of the metabolizable protein (MP) requirement model shows over-predictions of MP-allowable milk for 82% of the observations. The regression of residuals on predicted values has a slope bias of 0.34, that if subtracted from the assumed 67% efficiency results in an actual MP efficiency for milk protein synthesis of 44%. This value is in agreement with those reported in the literature (Doepel et al., 2004, Hanigan et al., 1998, Hristov et al., 2004).

Hanigan et al. (1998) pointed out that AA efficiencies in a reaction are always 100%. Therefore, MP efficiency for milk protein is determined by the rate of milk protein synthesis relative to the rates in the multiple pathways where AA are used. That means MP efficiency is determined by the factors that directly affect milk protein synthesis rate as well as the factors affecting the use of AA in other tissues, which determine the supply of AA to the mammary glands. Supply to the mammary glands is affected by splanchnic use of AA, and relative blood flow between splanchnic tissues and mammary gland.

In sheep, gut use of essential AA (EAA) has been shown to be proportional to supply, with more than 80%, except for Phe, extracted from arterial blood (peripheral recycling) rather than luminal absorption (MacRae et al., 1997). Conversely, liver used a fixed amount of branched chain AA (BCAA), Lys, and Thr, and amounts proportional to supply of His, Met, and Phe (Raggio et al., 2004). Using a modeling approach, Hanigan et al. (2004a) predicted 1% liver marginal removal of BCAA, 4% for Lys, and between 7 and 12% for other EAA when supply was increased by 10%. Liver removal of non-EAA

was more variable, ranging from 2% for Cys up to 70% for Asp. Therefore, splanchnic tissues extract most of the AA proportionally to supply, and the arterial supply is mainly recycling from peripheral circulation. Hence, if mammary extraction for a given AA increased, recycling to and use by splanchnic tissues would decrease (Hanigan et al., 2004b).

The other factor affecting supply of AA to the mammary glands is blood flow distribution. Both splanchnic and mammary blood flow positively respond to energy supply (Lomax and Baird, 1983, Rius et al., 2010, Rulquin et al., 2004). This benefits the mammary glands more because it has higher affinity for AA than splanchnic tissues (Hanigan et al., 1998). Splanchnic blood flow does not respond to AA supply (Hanigan et al., 2004b, Raggio et al., 2004). Total AA supply does not seem to have much effect on mammary blood flow either (Doepel and Lapierre, 2010). However, AA imbalance has been shown to significantly affect mammary blood flow and extraction of AA (Bequette et al., 1996, Bequette et al., 2000, Guinard and Rulquin, 1995). Despite being such a relevant factor on nutrient partitioning, little is known about blood flow regulation in the splanchnic tissues and mammary glands, and more research is needed on that topic.

Thus it is necessary to analyze how mammary cells respond to variable supplies of AA. Milk protein composition is stable through lactation (Ng-Kwai-Hang et al., 1982) and in response to diet manipulation (Grant and Patel, 1980). Caseins are the most abundant proteins in milk, representing more than 80% of all proteins present, followed by lactoglobulins and lactoalbumins (Grant and Patel, 1980). Among the caseins, α-S1 and ß are the most abundant (Bobe et al., 1999). Milk protein production is more variable

than composition, suggesting that factors affecting synthesis rates affect all the proteins, or at least the most abundant (α-S1 and ß caseins), similarlly.

Among factors reported to affect casein fractional synthesis rate (CFSR) are insulin (Bellacosa et al., 1998), which indicates systemic energy status, and intracellular AMP/ATP ratio (Hardie, 2004), which is a local indicator of energy status. These two factors have been shown to regulate protein synthesis through the mTOR pathway, which regulates mRNA translation initiation and elongation rates (Mahoney et al., 2009). Essential AA also have been shown to control mTOR phosphorylation and regulate protein synthesis in liver (O'Connor et al., 2004) and skeletal muscle (Davis et al., 2002) of neonatal pigs, and mammary gland tissue of lactating cows (Appuhamy et al., 2011a). This is expected because EAA are substrates for protein synthesis, and contrary to non-EAA, they cannot be synthesized by those tissues. Among EAA, the BCAA and specially Leu have the greatest effect on protein synthesis in skeletal muscle (Escobar et al., 2006, Suryawan et al., 2009) of neonatals pigs. In the liver, Leu did not show an effect in protein synthesis rate (Murgas Torrazza et al., 2010). Leucine has been shown to increase ß-lactoglobulin synthesis in mammary epithelial cells (Moshel et al., 2006) and Ile, Leu, Met, and Thr increased CFSR in mammary tissue (Appuhamy et al., 2012). In vivo, Leu alone had no effect on milk protein synthesis (Toerien et al., 2010). Methionine and Lys increased milk protein yield, but addition of Leu and Ile had no effect (Appuhamy, 2010). Other than the identification of the EAA with the greatest effect on milk protein synthesis, little is known about quantitative responses to those AA. Therefore, the objective of this study was to quantify the effect of Ile, Leu, Met, and Thr on casein syntheis in mammary tissue of lactating dairy cows, to determine the relation

between CFSR and protein signaling responses presented in chapther 3, and to explore interaction between these AA on CFSR responses.

METHODS

Tissue collection, treatment media preparation, tissue slices incubation and post-incubation processing were performed as described in chapter 3.

Intracellular Casein Enrichment

Caseins were precipitated from media and cell homogenate supernatants as described by Cuollo et al. (2010) with modifications. Briefly, samples were acidified by addition of a solution (1:1 v:v) with 1 mol/l acetic acid 1mol/l sodium acetate (pH 4.6), incubated for 30 min at 37ºC, and centrifuged 10 min at 4000 x *g*. Pellets were washed twice with ice cold 0.15 mol/l sodium acetate (pH 4.6), twice with ice cold acetone, and stored at -80ºC until analysis. Casein-enriched pellets were homogenized in 200 µl 500 mmol/l ammonium bicarbonate. Eighty nanograms of endoproteinase Glu-C (Sigma-Aldrich) in 180 µl 25 mM ammonium bicarbonate were added to each sample and then the samples were incubated over night at 37°C. The following day, 1.5 µl of each sample was spotted onto a MALDI target plate and allowed to air dry. Dried sample spots were then washed several times with 2 µl 0.1% trifluoroacetic acid (Sigma-Aldrich) in LC-MS grade water (Spectrum Chemical). Sample spots were then overlaid with 1 µl matrix solution which was then allowed to air dry. Matrix solution was 4 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) prepared in 50:50 LC-MS grade acetonitrile:LC-MS grade water (both from Spectrum Chemical) supplemented with 0.1% trifluoroacetic acid and 10 mmol/l ammonium chloride (both from Sigma-Aldrich). Spotted samples were then analyzed in reflector positive ion mode using a MALDI Tof/Tof analyzer (AB Sciex). A

spectrum was obtained for each sample for the m/z range of 1400 to 1500 summing 3000 laser shots. The MALDI control software 4000 series Data Explorer (AB Sciex) was used to export the cluster area values (total area of all isotopic peaks) for the alpha-S1-casein peptide 34-NLLRFFVAPFPE-45 (m/z = 1449.79) containing 0 (m+0), 2 (m+10), and 3 (m+15) labeled Phe. The cluster area corresponding to the peptide containing one labeled phenylalanine was negligible for all samples analyzed. $^{2}H_5$-Phe casein enrichment was not detected in medium samples, indicating that most of the casein synthesized during the incubation period remained within the tissue slice. Intracellular CFSR (%/h) was estimated as:

$$FSR_{(\%/h)} = \frac{100}{4} * \frac{\frac{m15}{E_{Phe\_tRNA}{}^{3}}}{\left(m0 - \frac{m15}{E_{Phe\_tRNA}{}^{3}} * \left(1 - E_{Phe\_tRNA}\right)^{3}\right)}$$

where E_{Phe_tRNA} represents the percent enrichment in the Phe acylated-transfer RNA (tRNA) pool, *m*0 represents the area under the curve for m+0, and m15 represents that for m+15. The numerator estimates labeled and unlabeled newly synthesized peptides based on a binomial distribution. The denominator estimates unlabeled peptides already present in the cells at the start of incubation. For this estimation, unlabeled newly synthesized peptides are subtracted from the unlabeled pool. Direct determination of AA-tRNA isotopic enrichment is tedious because it requires tRNA isolation and deacylation, and those steps can be compromised by contamination and losses (Baumann et al., 1994). Therefore, tRNA enrichment was assessed with an indirect method. The enrichment of a peptide with three Phe was determined by MALDI-TOF. The $tRNA^{Phe}$ enrichment equals the probability that a peptide with two $^{2}H_5$-Phe get a third $^{2}H_5$-Phe. As mRNA translation is a sequential process, only peptides that have the first and second Phe positions labeled

could have got a third $^{2}H_5$-Phe. Those peptides with two $^{2}H_5$-Phe, but one of those in the third position could never get three $^{2}H_5$-Phe. Furthermore, the probabilities of getting a $^{2}H_5$-Phe in any of the three positions are independent and equal. Therefore, $^{2}H_5$-Phe tRNA^{Phe} enrichment was indirectly estimated from the prevalence of m+15 and m+10 peptides:

$$E_{Phe\_tRNA} = \frac{m15}{m15 + \frac{m10}{3}}$$

where m10 is the area under the curve for m+10 peptides.

Statistical Analysis

The effects of Ile, Leu, Met, and Thr on $^{2}H_5$-Phe tRNA^{Phe} enrichment and CFSR were studied with a composite design as described by (Park et al., 1976) with modifications as explained in chapter 3. Briefly, a composite design consisting of a central point repeated 4 times, 2 axial points per AA, and a complete 2^4 factorial was used. The central points were set at 35% of Dulbecco's Modified Eagle Medium (DMEM) concentrations for the 4 AA studied. Axial points for each of the 4 AA were set at 0 and 100% of DMEM concentrations, holding the other 3 AA at 35% of DMEM concentration. Factorial points were set equidistant from central points at 20 and 50% of DMEM concentrations. Two additional treatments with all 4 AA at 0 or 100% DMEM were added to the experiment. The two additional treatments (0 and 100% of DMEM concentrations), plus central points, and factorial treatments with the 4 AA at 20 and 50% of DMEM concentrations were used to determine the response to the bulk of AA. The experiment was replicated in 5 cows. Data were analyzed with the following model:

$$y_{ijk} = \beta_0 + \sum_{i=1}^{4} \beta_{1_i} x_i + \sum_{i=1}^{4} \sum_{j=i}^{4} \beta_{2_{ij}} x_i x_j + z_k + e_{ijk}$$

$$z_k \sim IIDN(0, \sigma_z^2) \text{ and } e_{ijk} \sim IIDN(0, \sigma_e^2)$$

where y_{ijk} is the response of the i^{th} and j^{th} AA and k^{th} cow. x_i represents the concentration of each of the four EAA in the medium (mmol/l). β_0, β_1, and β_2 are the intercept, linear, and quadratic (quadratic plus interaction) parameter estimates, respectively; z_k represents the random effect of cow, and ε_{ik} the random error. Model parameters, least square estimates, standard errors, and confidence limits were estimated with the GLIMMIX procedure (SAS 9.3, SAS Institute Inc.). Model selection was performed backwards removing the least significant quadratic and one-way interaction term sequentially until all remaining terms left in the model were significant at $P < 0.1$. Non-significant linear terms that were not associated with any of the remaining quadratic terms were also removed. Root mean square prediction errors (RMSPE) were calculated for each model as described in chapter 3, and expressed as a percent of the mean. Mean (MB) and slope bias (SB) were expressed as percent of the mean square prediction error (chapter 3).

RESULTS

Increasing concentrations of Ile, Leu, Met, and Thr had no effect on Phe-tRNA enrichment (Table 4.1). However, CFSR increased curvilinearly in response to these 4 AA (Table 4.1 and Figure 4.1), with an estimated maximum response at 63% of DMEM concentrations and a predicted response of 4.32 ± 0.84 %/h. The response at DMEM concentrations did not differ from that observed at 50% of DMEM concentrations, or at the maximum (95% confidence limit). Therefore, it seems like mammary cells responded curvilinearly to these AA reaching a plateau. The observed response in protein synthesis

was similar to that observed for mTOR signaling (chapter 3), supporting the modification made to the classical central composite design. For this study, the "central" point was not set equidistant from the axial points. This ensured fewer points in the plateau, and more in the curvilinear region. However, with this approach the design lost rotatability and orthogonality of the parameter estimates (St-Pierre and Weiss, 2009). The expected error at 100% is larger than that at 0% of DMEM concentrations, and predicted CFSR at DMEM concentrations should be considered with some caution. As parameters estimates are not independent, model selection had to be done backwards, as explained.

The fact that mammary cells responded detrimentally to AA supply suggests that in vivo more AA will not always mean more milk protein. As supply of AA increases, a smaller proportion of the AA that reaches the glands will be extracted and used for protein synthesis (Whitelaw et al., 1986). A large proportion of absorbed AA reaches peripheral circulation (MacRae et al., 1997). However, not all the AA that escape first pass splanchnic catabolism and reach the mammary glands are used for protein synthesis. This study shows that as supply of Ile, Leu, Met, and Thr combined increases the proportion used for milk protein synthesis decreases, and at some point the marginal use of extra supply can reach zero. If that is the case in vivo, then mammary extraction efficiency would decrease, and more AA would return to circulation. Splanchnic blood flow is 3 to 4 times that of the mammary glands (Hanigan et al., 2004b, Raggio et al., 2004), and most of the splanchnic use of AA corresponds to arterial supply. Furthermore, splanchnic use of AA is proportional to supply (Hanigan et al., 2004b, MacRae et al., 1997). Therefore, MP efficiency for milk protein would decrease with protein supply, and AA catabolism by splanchnic tissues and urea production would increase. Variable

MP efficiency agrees with variable animal responses to MP supply reported in the literature (Doepel et al., 2004, Hanigan et al., 1998, Hristov et al., 2004), and is not consistent with the use of a fixed MP partial efficiency for milk protein synthesis as adopted by most nutrient requirement systems. By adopting a fixed partial efficiency, models over-predict responses as the system approaches the plateau, and this region is close to maximum requirement, for which most rations are formulated (Hanigan et al., 1998).

Individual AA Effects on Fractional Synthesis Rate of Casein

The CFSR model included linear and quadratic terms. The one-way interaction between Ile and Thr tended to be significant, and was on the borderline of the criteria ($P < 0.1$) to remain in the model (Table 4.2). If the model were orthogonal, removal of insignificant independent variables should not change the other parameter estimates. However, as noted above, the design used for this work was not orthogonal (St-Pierre and Weiss, 2009). The correlation between the Ile × Thr interaction and the linear parameters was -0.78 (Ile) and -0.79 (Thr). Removing the interaction from the model increased the Ile estimate by 3.5 fold and the Thr estimate by 2.5 fold (Table 4.2) and reduced the correlation between Ile and Thr from 0.68 to 0.18. These results indicate that the interaction explained very little of the variation, and may be fitting a few potential outliers. Therefore, the decision was made to remove the Ile × Thr interaction from the equation, and the rest of the analysis was based on the model with only linear and quadratic terms.

Prediction errors of CFSR were large (31.8 %), but they did not exhibit mean or slope bias. The maximum responses to each of the 4 AA were at 71, 49, 60, and 65% of

DMEM for Ile, Leu, Met, and Thr respectively (Figure 4.2). Those values correspond to 270, 120, 440, and 140% the plasma concentrations of Ile, Leu, Met, and Thr, respectively, observed in a lactating cow fed a 16% CP diet (Appuhamy et al., 2011b). The CFSR estimated at those maximums were similar (95% CL) between AA (3.6 ± 0.6 %/h). The CFSR observed for Ile, Met, and Thr at DMEM concentrations did not differ from the maximum response estimated for those AA and from those observed at 50% of DMEM concentrations, suggesting that the CFSR response curve started bending at low concentrations and then plateaued somewhere before the maximum reported for each AA. The same was not observed for Leu in which the response at DMEM concentration was lower than those observed at 50% of DMEM concentration. Deleterious effects of AA on CFSR have been previously reported, but not for Leu (Prizant and Barash, 2008). A potential explanation for a decrease in CFSR responses to Leu after the maximum could be that as the response to Leu reached a plateau somewhere before 50% of DMEM concentration, from there to DMEM concentration, addition of Leu could have inhibited the transport of other AA that share the same transport system (e.g. Ile). Intracellular concentrations of Ile showed a numerical decrease when compared to Leu concentrations at 0 and 100% of DMEM (data not showed). In other treatments Ile was intentionally manipulated, so they could not be used for the analysis. It is worth notice that Leu, was the AA with the maximum response reached at the lowest concentration as a percent of DMEM. Therefore, more of the Leu response curve was in the saturated region with no response in CFSR. Previous studies that found an effect of Leu up to DMEM concentrations in mammary tissue used a deletion approach, in which the other EAA remained at DMEM concentrations, which may have prevented changes in AA transport

from being observed (Appuhamy et al., 2012). In fact, the decrease in CFSR observed in response to Leu, was not observed when Ile, Met, and Thr were also increased in the medium (Figure 4.2).

All interactions between AA were non-significant (Ile × Thr was already discussed) and thus were removed from the model. That means that the model predicts the same incremental responses to Leu, Met, or Thr at any concentration of Ile, and vice versa (i.e. additive effects, Figure 4.3). The type of response predicted by this model at the tissue level contradicts current concepts in nutrient requirement systems. Dairy nutrient requirement systems are based on the single-limiting AA theory (Mitchell and Block, 1946). This theory was brought to animal nutrition from plant nutrition, and establishes that a response can only be observed if the nutrient that is most limiting relative to requirements is provided (Sprengel, 1828, von Liebig, 1863). Most of the individual nutrient studies in dairy cattle are not designed to challenge this theory. However, Park et al. (1976) studied the responses of CFSR in mammary tissue of lactating rats and observed that the interactions between three groups of EAA were not significant. Non-significant interactions indicate additive effects between groups of AA and violate the single-limiting AA theory. Hanigan et al. (2000) observed that curvilinear additive responses to individual AA explained more variation than a single-limiting AA modeling approach. This observation is in agreement with the model fitted in the present study where independent curvilinear responses to individual AA can be predicted. This theory has also been challenged with respect to energy and protein additive effects on protein synthesis (Appuhamy et al., 2011a, Rius et al., 2010).

The fact that the mammary glands respond additively to individual AA is significant to diet formulation because, one could increase the concentration of specific AA (e.g. Ile, Leu) and obtain a response in protein synthesis, i.e. in N partial efficiency. Otherwise, one could reduce CP supply and supplement with individual AA that specifically stimulate milk protein synthesis to obtain similar levels of milk protein production, reducing markedly N excretion to the environment. These findings also challenge current nutrient requirement systems because they cannot accommodate a response in milk protein to a correction in the post-absorptive AA profile, but for Met and Lys. Similarly, current systems are not designed to follow a strategy in diet formulation, as just mentioned, where dietary CP level is reduced and specific AA are supplemented to stimulate the rate of protein synthesis.

Translation Regulation and Fractional Synthesis Rate of Casein

Surprisingly, CFSR responses were not correlated with phosphorylation state of signaling proteins presented in chapter 3 (Table 4.3). These results contradict previous reports that observed a positive correlation between mTOR activation and CFSR of protein in mammary tissue (Appuhamy et al., 2011a, Appuhamy et al., 2012). In those studies tissue slices were incubated for 75 min versus the 4 h used in the present study. Longer incubation time could have reduced phosphorylation of proteins and affected the correlation with CFSR. However, in this study mTOR positively responded to Ile and Leu signaling. Furthermore, previous studies in pigs also showed mTOR activation and increased protein synthesis rate in skeletal muscle after 4 h infusion of an AA mix (Davis et al., 2002).

Another reason could be the non-linear responses to AA captured in the present study. Isoleucine stimulated mTOR and rpS6 phosphorylation linearly (chapter 3). In agreement with previous observations (Appuhamy et al., 2012), Ile stimulated CFSR. However, this study observed a decrease in the response to Ile after about 0.25 mmol/l of medium, whereas the activation of mTOR pathway continued up to 0.42 mmol of Ile/l of medium (DMEM concentration). Therefore, there is a large region where cell signaling increased and there was no response in CFSR. The response to Ile observed in CFSR could be explained by substrate effect. However, the mismatch between mTOR activation and CFSR at higher concentrations of Ile raises the question of why protein synthesis reached a plateau when mTOR continued stimulating protein synthesis. It is not likely that other EAA that remained at DMEM concentrations limited protein synthesis. Methionine and Thr increased CFSR similar to Ile and Leu, but they did not cause the same effect on signaling molecules. Therefore, part of the variation observed in CFSR in response to Met and Thr does not correlate with protein signaling. Further studies are required to explain those results, and we can only speculate that as CFSR is much higher than protein synthesis rates in other tissues, the translation machinery is fully activated during lactation and the responses to mTOR signaling could be milder than in e.g. muscle. Thus, it would be substrate availability a potential limitation for milk protein synthesis in the mammary glands. This hypothesis is in agreement with the increase in $tRNA^{Phe}$ enrichment in response to Met, but not to the other AA studied. A similar study that analyzed non-milk mammary protein responses in lactating or non-lactating tissue could help to better understand these results.

Individual AA Effects on tRNA Enrichment

The model fitted to predict individual AA effects on tRNA enrichment included linear effects of Ile, Met, and Thr, quadratic effect of Met, and one-way Ile and Thr interaction (Table 4.2). Residual errors were minimal (< 2%), and they did not display mean or slope bias. Such low errors could be partially explained by the minimal variation observed in $tRNA^{Phe}$ enrichment. The estimate of the intercept was 0.961 ± 0.013 ($P <$ 0.001), indicating that most of the tRNA bound Phe were labeled. This was expected because media did not include any unlabeled Phe. Thus, the sources of unlabeled Phe were the intracellular free pool, which is very small and would be further reduced by equilibration with the extracellular pool, and release of Phe from protein degradation. Tyrosine hydroxylase activity was disregarded in the tissue because $^{2}H_4$-Tyr enrichment was undetected. Hanigan et al. (2009) reported transamination of Phe in the mammary gland, but this did not represent a significant lost of the AA because the ^{13}C labeling was conserved. Thus, a change in $tRNA^{Phe}$ enrichment could only be explained by changes in the protein synthesis-degradation balance. Assuming that individual AA have no effect on protein degradation (reports on the contrary were not found), a change on $tRNA^{Phe}$ enrichment would be an indicator of changes in CFSR. Increasing CFSR would reduce the intracellular Phe pool, and more AA would be transported into the cell. As mentioned, culture media lacked unlabeled Phe, thus, for Phe an increase on CFSR would mean an increase in intracellular Phe enrichment, and hence, $tRNA^{Phe}$ enrichment. However, if that were the case $tRNA^{Phe}$ enrichment would be positively correlated with CFSR, but that was not the case, $tRNA^{Phe}$ enrichment had no correlation with CFSR (data not

shown). That observation raises the question if protein degradation is actually affected by intracellular concentrations of AA, and the assumption made above is not correct.

Isoleucine linearly decreased $^{2}H_5$-Phe enrichment of the $tRNA^{Phe}$ ($P = 0.046$). If CFSR affected $tRNA^{Phe}$ enrichment, these results would contradict the ones reported above where Ile increased CFSR, and would suggest a negative effect of Ile on CFSR. Thr inhibited Ile effect on $tRNA^{Phe}$ enrichment (Ile × Thr, $P = 0.086$), and this is in agreement to what was observed in CFSR. Increasing Thr concentration from 20 to 50% of DMEM concentration negated the Ile effect on $E_{Phe\text{-}tRNA}$ (Figure 4.4). Met caused a curvilinear response in $E_{Phe\text{-}tRNA}$ (Figure 4.5), being the response at 50% of DMEM concentration significantly higher than the observed with no Met, and no response was observed after that concentration. Leu had no effect on $tRNA^{Phe}$ enrichment, and thus parameters were removed from the equation.

CONCLUSIONS

The saturable independent responses to individual EAA in CFSR observed in this study contradict the single-limiting AA theory assumed in current requirement systems and demonstrates deficiencies in a fixed post-absorptive AA efficiency approach for determining milk protein synthesis requirements. These concepts should help to improve milk protein predictions in future nutrient requirement models.

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REFERENCES

Appuhamy, J. A. D. R. N. 2010. Regulatory roles of essential amino acids, energy, and insulin in mammary cell protein synthesis. in Animal Science, Dairy. Vol. Doctor of Philosophy. Virginia Polytechnic Institute and State University, Blacksburg, VA.

Appuhamy, J. A. D. R. N., A. L. Bell, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2011a. Essential amino acids regulate both initiation and elongation of mrna translation independent of insulin in mac-t cells and bovine mammary tissue slices. J. Nutr. 141(6):1209-1215.

Appuhamy, J. A. D. R. N., J. R. Knapp, O. Becvar, J. Escobar, and M. D. Hanigan. 2011b. Effects of jugular-infused lysine, methionine, and branched-chain amino acids on milk protein synthesis in high-producing dairy cows. J. Dairy Sci. 94(4):1952-1960.

Appuhamy, J. A. D. R. N., N. A. Knoebel, W. A. D. Nayananjalie, J. Escobar, and M. D. Hanigan. 2012. Isoleucine and leucine independently regulate mtor signaling and protein synthesis in mac-t cells and bovine mammary tissue slices. J. Nutr. 142(3):484-491.

Baker, D. H. 1996. Advances in amino acid nutrition and metabolism of swine and poultry. Pages 41-52 in Nutrient managment of food animals to enhance and protect the environment. E. T. Kornegay, ed. CRC Lewis, Boca Raton, FL.

Baumann, P. Q., W. S. Stirewalt, B. D. O'Rourke, D. Howard, and K. S. Nair. 1994. Precursor pools of protein synthesis: A stable isotope study in a swine model. Am. J. Physiol. Endocrinol. 267(2):E203-E209.

Bellacosa, A., T. O. Chan, N. N. Ahmed, K. Datta, S. Malstrom, D. Stokoe, F. McCormick, J. Feng, and P. Tsichlis. 1998. Akt activation by growth factors is a multiple-step process: The role of the ph domain. Oncogene 17(3):313-325.

Bequette, B. J., F. R. C. Backwell, J. C. MacRae, G. E. Lobley, L. A. Crompton, J. A. Metcalf, and J. D. Sutton. 1996. Effect of intravenous amino acid infusion on leucine oxidation across the mammary gland of the lactating goat. J. Dairy Sci. 79(12):2217-2224.

Bequette, B. J., M. D. Hanigan, A. G. Calder, C. K. Reynolds, G. E. Lobley, and J. C. MacRae. 2000. Amino acid exchange by the mammary gland of lactating goats when histidine limits milk production. J. Dairy Sci. 83(4):765-775.

Bobe, G., D. C. Beitz, A. E. Freeman, and G. L. Lindberg. 1999. Effect of milk protein genotypes on milk protein composition and its genetic parameter estimates1. J. Dairy Sci. 82(12):2797-2804.

Cuollo, M., S. Caira, O. Fierro, G. Pinto, G. Picariello, and F. Addeo. 2010. Toward milk speciation through the monitoring of casein proteotypic peptides. Rapid Commun. Mass Spectrom. 24(11):1687-1696.

Davis, T. A., M. L. Fiorotto, D. G. Burrin, P. J. Reeds, H. V. Nguyen, P. R. Beckett, R. C. Vann, and P. M. J. O'Connor. 2002. Stimulation of protein synthesis by both insulin and amino acids is unique to skeletal muscle in neonatal pigs. Am. J. Physiol. Endocrinol. Metab. 282(4):E880-890.

Doepel, L., D. Pacheco, J. J. Kennelly, M. D. Hanigan, I. F. López, and H. Lapierre. 2004. Milk protein synthesis as a function of amino acid supply. J. Dairy Sci. 87(5):1279-1297.
Doepel, L. and H. Lapierre. 2010. Changes in production and mammary metabolism of dairy cows in response to essential and nonessential amino acid infusions. J. Dairy Sci. 93(7):3264-3274.
Escobar, J., J. W. Frank, A. Suryawan, H. V. Nguyen, S. R. Kimball, L. S. Jefferson, and T. A. Davis. 2006. Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs. Am. J. Physiol. Endocrinol. 290(4):E612-E621.
Grant, D. R. and P. R. Patel. 1980. Changes of protein composition of milk by ratio of roughage to concentrate. J. Dairy Sci. 63(5):756-761.
Guinard, J. and H. Rulquin. 1995. Effects of graded amounts of duodenal infusions of methionine on the mammary uptake of major milk precursors in dairy cows. J. Dairy Sci. 78(10):2196-2207.
Hanigan, M. D., J. P. Cant, D. C. Weakley, and J. L. Beckett. 1998. An evaluation of postabsorptive protein and amino acid metabolism in the lactating dairy cow. J. Dairy Sci. 81(12):3385-3401.
Hanigan, M. D., J. France, L. A. Crompton, and B. J. Bequette. 2000. Evaluation of a representation of the limiting amino acid theory for milk protein synthesis. in Modelling nutrient utilization in farm animals. J. P. McNamara, J. France, and D. E. Beever, ed. CABI, Wallingford.
Hanigan, M. D., L. A. Crompton, C. K. Reynolds, D. Wray-Cahen, M. A. Lomax, and J. France. 2004a. An integrative model of amino acid metabolism in the liver of the lactating dairy cow. J. Theor. Biol. 228(2):271-289.
Hanigan, M. D., C. K. Reynolds, D. J. Humphries, B. Lupoli, and J. D. Sutton. 2004b. A model of net amino acid absorption and utilization by the portal-drained viscera of the lactating dairy cow. J. Dairy Sci. 87(12):4247-4268.
Hanigan, M. D., J. France, S. J. Mabjeesh, W. C. McNabb, and B. J. Bequette. 2009. High rates of mammary tissue protein turnover in lactating goats are energetically costly. J. Nutr. 139(6):1118-1127.
Hardie, D. G. 2004. The amp-activated protein kinase pathway - new players upstream and downstream. J. Cell Sci. 117(23):5479-5487.
Hristov, A. N., W. J. Price, and B. Shafii. 2004. A meta-analysis examining the relationship among dietary factors, dry matter intake, and milk and milk protein yield in dairy cows. J. Dairy Sci. 87(7):2184-2196.
Lomax, M. A. and G. D. Baird. 1983. Blood flow and nutrient exchange across the liver and gut of the dairy cow. Br. J. Nutr. 49(03):481-496.
MacRae, J. C., L. A. Bruce, D. S. Brown, and A. G. Calder. 1997. Amino acid use by the gastrointestinal tract of sheep given lucerne forage. American Journal of Physiology - Gastrointestinal and Liver Physiology 273(6):G1200-G1207.
Mahoney, S. J., J. M. Dempsey, and J. Blenis. 2009. Chapter 2 cell signaling in protein synthesis: Ribosome biogenesis and translation initiation and elongation. Pages 53-107 in Progress in molecular biology and translational science. Vol. Volume 90. W. B. H. John, ed. Academic Press.

Mitchell, H. H. and R. J. Block. 1946. Some relationships between the amino acid contents of proteins and their nutritive values for the rat. J. Biol. Chem. 163(3):599-620.
Moshel, Y., R. E. Rhoads, and I. Barash. 2006. Role of amino acids in translational mechanisms governing milk protein synthesis in murine and ruminant mammary epithelial cells. J. Cell. Biochem. 98(3):685-700.
Murgas Torrazza, R., A. Suryawan, M. C. Gazzaneo, R. n. A. Orellana, J. W. Frank, H. V. Nguyen, M. L. Fiorotto, S. El-Kadi, and T. A. Davis. 2010. Leucine supplementation of a low-protein meal increases skeletal muscle and visceral tissue protein synthesis in neonatal pigs by stimulating mtor-dependent translation initiation. J. Nutr.
Ng-Kwai-Hang, K. F., J. F. Hayes, J. E. Moxley, and H. G. Monardes. 1982. Environmental influences on protein content and composition of bovine milk. J. Dairy Sci. 65(10):1993-1998.
NRC. 2001. Nutrient requirements of dairy cattle. 7th ed. National Academy Press, Washington, DC.
O'Connor, P. M. J., S. R. Kimball, A. Suryawan, J. A. Bush, H. V. Nguyen, L. S. Jefferson, and T. A. Davis. 2004. Regulation of neonatal liver protein synthesis by insulin and amino acids in pigs. Am. J. Physiol. Endocrinol. 286(6):E994-E1003.
Park, C. S., P. T. Chandler, and R. M. Clark. 1976. Optimum amino acid complement for protein synthesis by rat mammary cells in tissue culture1. J. Dairy Sci. 59(10):1758-1763.
Prizant, R. L. and I. Barash. 2008. Negative effects of the amino acids lys, his, and thr on s6k1 phosphorylation in mammary epithelial cells. J. Cell. Biochem. 105(4):1038-1047.
Raggio, G., D. Pacheco, R. Berthiaume, G. E. Lobley, D. Pellerin, G. Allard, P. Dubreuil, and H. Lapierre. 2004. Effect of level of metabolizable protein on splanchnic flux of amino acids in lactating dairy cows. J. Dairy Sci. 87(10):3461-3472.
Rius, A. G., J. A. D. R. N. Appuhamy, J. Cyriac, D. Kirovski, O. Becvar, J. Escobar, M. L. McGilliard, B. J. Bequette, R. M. Akers, and M. D. Hanigan. 2010. Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. J. Dairy Sci. 93(7):3114-3127.
Rulquin, H., S. Rigout, S. Lemosquet, and A. Bach. 2004. Infusion of glucose directs circulating amino acids to the mammary gland in well-fed dairy cows. J. Dairy Sci. 87(2):340-349.
Sprengel, C. 1828. Von den substanzen der ackerkrume und des untergrundes (on the substances in the topsoil and the subsoil). Journal für technische und ökonomische Chemie 2:423-474.
St-Pierre, N. R. and W. P. Weiss. 2009. Technical note: Designing and analyzing quantitative factorial experiments1. J. Dairy Sci. 92(9):4581-4588.
Suryawan, A., P. M. J. O'Connor, J. A. Bush, H. V. Nguyen, and T. A. Davis. 2009. Differential regulation of protein synthesis by amino acids and insulin in peripheral and visceral tissues of neonatal pigs. Amino Acids 37(1):97-104.
Toerien, C. A., D. R. Trout, and J. P. Cant. 2010. Nutritional stimulation of milk protein yield of cows is associated with changes in phosphorylation of mammary

eukaryotic initiation factor 2 and ribosomal s6 kinase 1. J. Nutr. 140(2):285-292.
von Liebig, J. 1863. The natural laws of husbandry. Walton & Maberly, London.
Whitelaw, F. G., J. S. Milne, E. R. Ørskov, and J. S. Smith. 1986. The nitrogen and energy metabolism of lactating cows given abomasal infusions of casein. Br. J. Nutr. 55(03):537-556.
Wolfe, A. H. and J. A. Patz. 2002. Reactive nitrogen and human health:Acute and long-term implications. Ambio 31(2):120-125.

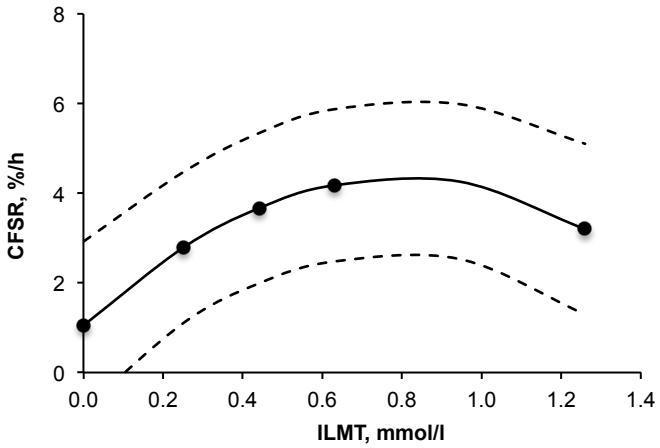
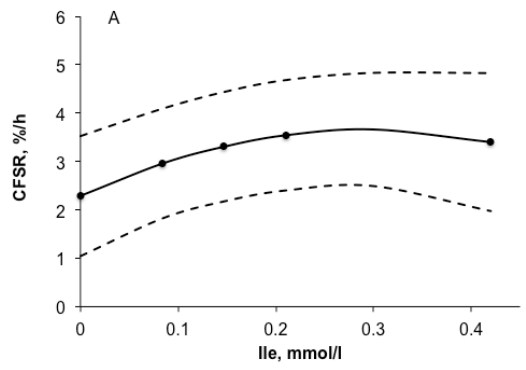
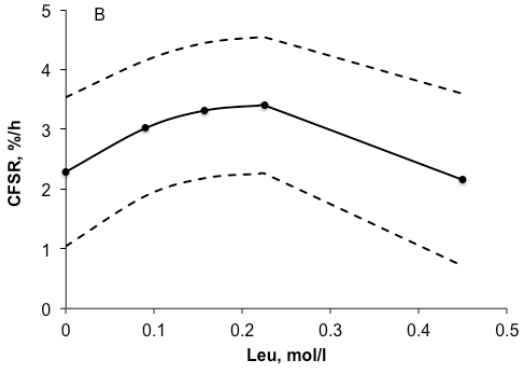
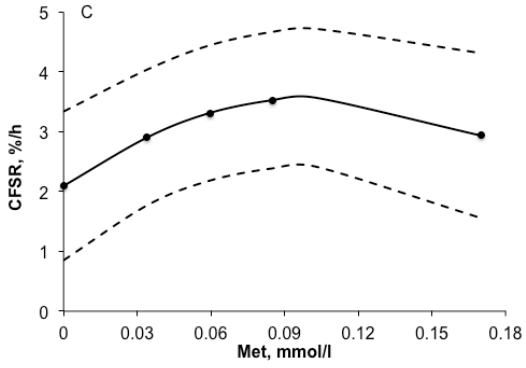

8
6
4
2
0
CFSR, %/h
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
ILMT, mmol/l

Figure 4.1. α-S1 casein fractional synthesis rate (CFSR, %/h) responses to Ile, Leu, Met, and Thr supply.
The solid line represents the fractional synthesis rate responses (%/h), dashed lines represent 95% confidence limits, and markers represent least square estimates at 0, 20, 35, 50, and 100% of Dulbecco's Modified Eagle Medium (DMEM) concentrations for Ile, Leu, Met, and Thr. Other AA remained at DMEM concentrations. ILMT: Ile, Leu, Met, Thr.


A
CFSR, %/h
Ile, mmol/l
0
1
2
3
4
5
6
0
0.1
0.2
0.3
0.4


B
CFSR, %/h
Leu, mol/l
0
1
2
3
4
5
0
0.1
0.2
0.3
0.4
0.5


C
CFSR, %/h
Met, mmol/l
0
1
2
3
4
5
0
0.03
0.06
0.09
0.12
0.15
0.18

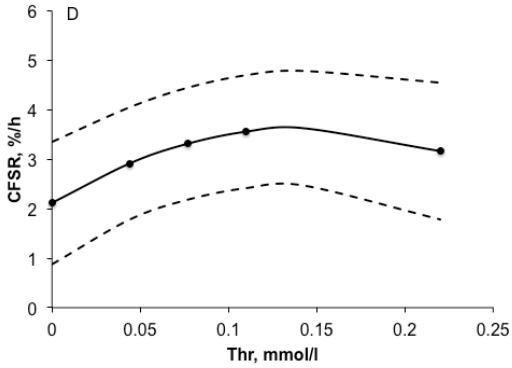

D
CFSR, %/h
Thr, mmol/l
0
1
2
3
4
5
6
0
0.05
0.1
0.15
0.2
0.25

Figure 4.2. α-S1 casein fractional synthesis rate responses (FSR, %/h) to media concentrations of Ile (A), Leu (B), Met (C), and Thr (D).
Solid lines indicate predicted values by the CFSR complete model presented in table 2. Dashed lines represent 95% confident limits. Markers represent least square estimates for concentrations of Ile (A), Leu (B), Met (C), and Thr (D) at 0, 20, 35, 50, and 100% of Dulbecco's Modified Eagle Medium (DMEM). The other three AA studied were held at 35% of DMEM concentrations. Estimates at 50% of DMEM concentrations were significantly higher than at 0% of DMEM concentrations for all AA but Leu. Estimates at DMEM concentrations did not differ from those at 50% of DMEM concentrations for any AA but Leu.

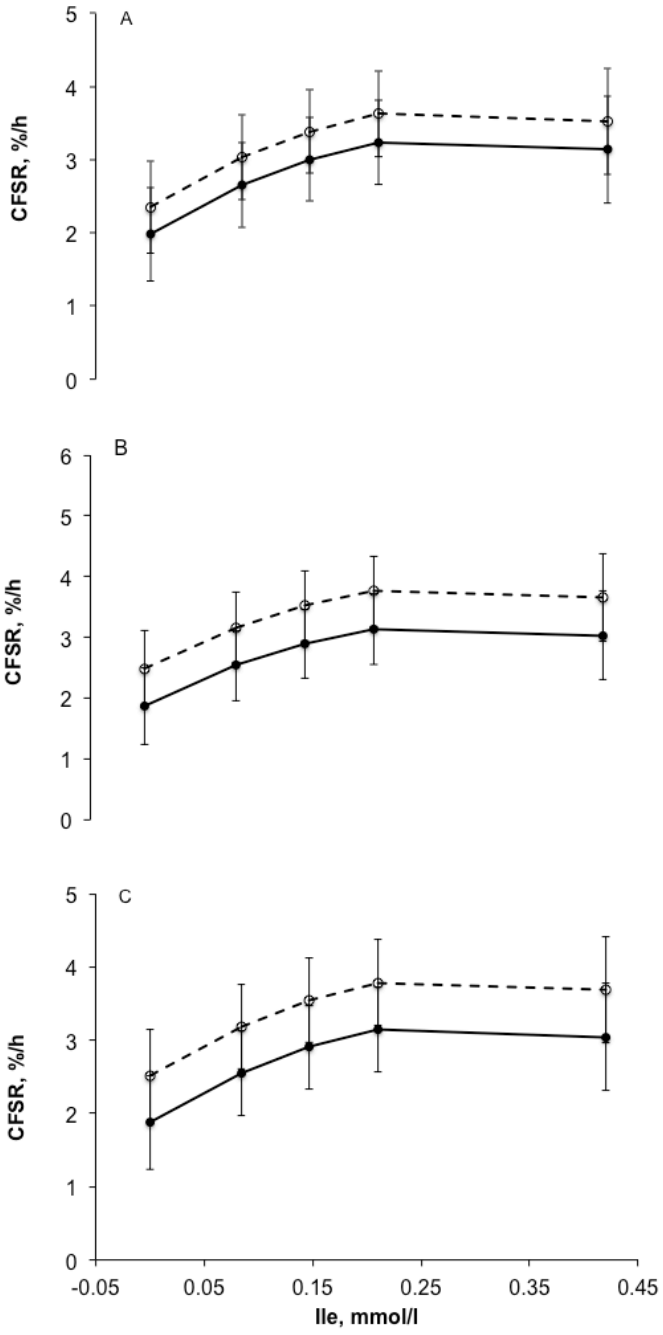

A
B
C
CFSR, %/h
CFSR, %/h
CFSR, %/h
Ile, mmol/l
-0.05
0.05
0.15
0.25
0.35
0.45

Figure 4.3. α-S1 casein fractional synthesis rate responses (CFSR, %/h) to media concentrations of Ile with Leu (A), Met (B), or Thr (C) at 20 (solid line) or 50% of Dulbecco's Modified Eagle Medium (DMEM; dashed line).
The other two AA studied were held at 35% of DMEM concentrations. Circles correspond with Ile concentrations at 0, 20, 35, 50, and 100% of DMEM concentrations.

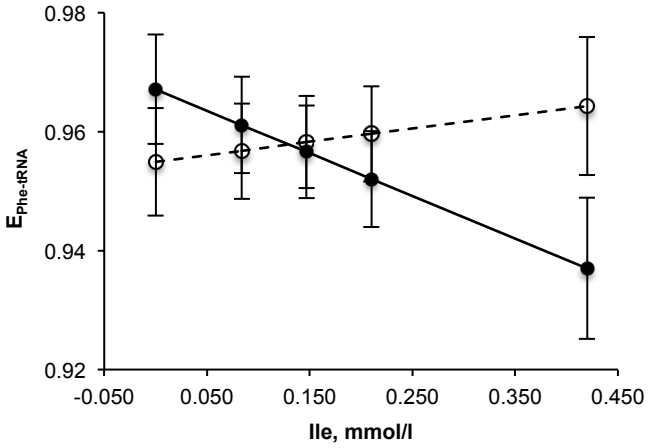

0.98
0.96
0.94
0.92
$E_{Phe\text{-}tRNA}$
-0.050
0.050
0.150
0.250
0.350
0.450
Ile, mmol/l

Figure 4.4. $^{2}H_5$ Phe $tRNA^{Phe}$ enrichment ($E_{Phe\text{-}tRNA}$) responses to Ile with Thr at 20% (solid line) and 50% of Dulbecco's Modified Eagle Medium (DMEM) concentrations (dashed line).
Markers represent least square estimates of Ile concentrations at 0, 20, 35, 50, and 100% of DMEM concentrations. Leu and Met were held at 35% of DMEM concentrations.

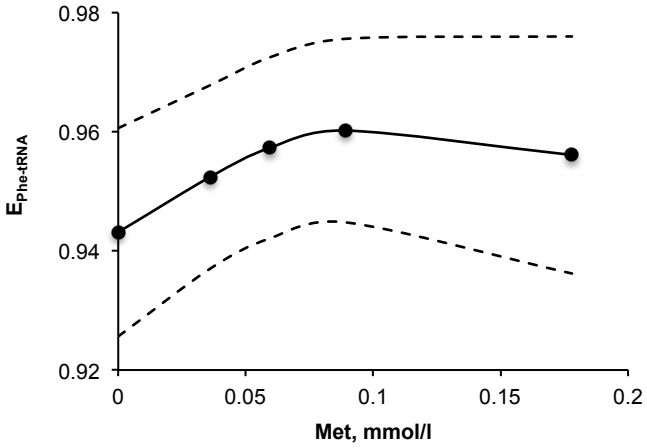

0.98
0.96
0.94
0.92
$E_{Phe\text{-}tRNA}$
0
0.05
0.1
0.15
0.2
Met, mmol/l

Figure 4.5. $^{2}H_5$ Phe $tRNA^{Phe}$ enrichment ($E_{Phe\text{-}tRNA}$) responses to Met (solid lane). Markers represent least square estimates of Met concentrations at 0, 20, 35, 50, and 100% of Dulbecco's Modified Eagle Medium (DMEM). Dashed lines represent 95% confidence limits. Ile, Leu, and Thr were held at 35% of DMEM concentrations.

Table 4.1. Parameter estimates and significance level for the effect of Ile, Leu, Met, and Thr on $^{2}H_5$Phe-tRNA^{Phe} enrichment ($E_{Phe\text{-}tRNA}$) and α-S1 casein fractional synthesis rate (CFSR, %/h).

	$E_{Phe\text{-}tRNA}$			CFSR		
Effect	Estimate	SE	*P*	Estimate	SE	*P*
Intercept	0.95	0.009	<0.001	1.05 (h^{-1})	0.92	0.3
ILMT[1]	0.01 (l/mol)	0.010	0.4	8.21 (l/mmol/h)	1.54	<0.001
ILMT × ILMT				-5.16 (l^2/$mmol^2$/h)	1.09	<0.001
RMSE % mean[2]				25.9		
MB % MSE[3]				0.0		
SB % MSE[4]				0.4		

[1]ILMT: combined effect of Ile, Leu, Met, and Thr. Included only treatments with all 4 AA at 0, 20, 35, 50, and 100% of Dulbecco's Modified Eagle Medium concentrations
[2]RMSPE % mean: root mean square prediction errors expressed as percent of the sample mean
[3]MB % MSE: mean bias expressed as percent of the mean square prediction errors
[4]SB % MSE: slope bias expressed as percent of the mean square prediction errors

Table 4.2. Model selection for Ile, Leu, Met, and Thr effects on $^{2}H_5$Phe-tRNA^{Phe} enrichment ($E_{Phe\text{-}tRNA}$) and α-S1 casein fractional synthesis rate (CFSR, %/h).

	$E_{Phe\text{-}tRNA}$			CFSR					
Effect	Estimate	SE	*P*	Estimate-1	SE	*P*	Estimate-2[1]	SE	*P*
Intercept	0.961	0.013	<0.001	-0.17 (h^{-1})	1.05	0.9	-1.16 (h^{-1})	0.87	0.25
ILE	-0.134 (l/mol)	0.067	0.046	2.63 (l/mmol/h)	5.10	0.6	9.30 (l/mmol/h)	3.23	0.005
LEU				10.16 (l/mmol/h)	3.00	0.001	10.16 (l/mmol/h)	3.02	0.001
MET	0.326 (l/mol)	0.120	0.008	28.68 (l/mmol/h)	7.79	<0.001	28.71 (l/mmol/h)	7.84	<0.001
THR	-0.185 (l/mol)	0.126	0.14	8.63 (l/mmol/h)	9.60	0.4	21.34 (l/mmol/h)	5.98	0.001
ILE × ILE				-15.82 (l^2/$mmol^2$/h)	7.86	0.046	-15.53 (l^2/$mmol^2$/h)	7.92	0.052
LEU × LEU				-23.27 (l^2/$mmol^2$/h)	6.89	0.001	-23.27 (l^2/$mmol^2$/h)	6.94	0.001
MET × MET	-1.466 (l^2/mol^2)	0.697	0.038	-139.66 (l^2/$mmol^2$/h)	45.79	0.003	-140.09 (l^2/$mmol^2$/h)	46.12	0.003
THR × THR				-75.54 (l^2/$mmol^2$/h)	27.06	0.006	-74.95 (l^2/$mmol^2$/h)	27.26	0.007
ILE × THR	1.424 (l^2/mol^2)	0.822	0.086	87.00 (l^2/$mmol^2$/h)	51.65	0.095			
RMSE % mean[2]	1.55			31.5			31.8		
MB % MSE[3]	0.0			0.0			0.0		
SB % MSE[4]	0.1			0.0			0.1		

[1]Model fitted without Ile × Thr. Interaction removed because of high correlation with linear parameters
[2]RMSPE %: root mean square prediction error expressed as percent of the observed mean
[3]MB %: mean bias expressed as percent of the mean square prediction error
[4]SB %: slope bias expressed as percent of the mean square prediction error

Table 4.3. Pearson correlations and probabilities between α-S1 casein fractional synthesis rate (CFSR, %/h) and phosphorylation state of signaling proteins.

		Phosphorylated to total ratio			
		eIF2α[1]	**mTOR[2]**	**eEF2[3]**	**rpS6[4]**
CFSR	Corr[5]	-0.04	0.06	-0.004	-0.05
	P	0.7	0.5	1.0	0.6

[1]eIF2α: Eukaryotic initiation factor 2 α subunit
[2]mTOR: Mammalian target of rapamycin
[3]eEF2: Eukaryotic elongation factor 2
[4]rpS6: Ribosomal protein S6
[6]Corr: Pearson correlation

CHAPTER 5: SUMMARY

Dairy cattle only capture 25% of dietary N in milk, and excrete most of the remaining N to the environment. About two thirds of N losses happen post absorption, when absorbed AA are catabolized by splanchnic tissues and the N is converted to urea. In order to understand how these losses happen, and to look for strategies that could mitigate them, current concepts in post-absorptive metabolism of AA were reviewed. Recent research demonstrated that splanchnic tissues have low affinity for most of the AA as compared with the mammary glands. Therefore, most of the AA absorbed from the lumen of the small intestine escape splanchnic tissues and reach peripheral circulation. Based on conservation of mass principles, amino acid efficiency in post-absorptive reactions (e.g. protein synthesis) is 100%. Then, post-absorptive N efficiency of AA for milk protein synthesis depends on the proportion of AA that reaches the mammary glands, and the proportion of supply that the glands extract and use for milk protein synthesis. Relative supply is a function of relative blood flow. In the last few years, splanchnic and mammary studies have demonstrated that blood flow to those tissues can be modified by nutrient manipulation. Despite the lack of a complete understanding of the regulatory mechanisms, enough data on splanchnic and mammary regulation of blood flow have been generated to incorporate these concepts into AA metabolism models. The second parameter in the equation is how much of the supplied AA are extracted. It has been shown that swine can use absorbed AA with efficiencies up to 85%. Mammary extraction of AA in cows fed common US dairy diets (18% CP) is significantly lower. Amino acids not extracted by the gland return to peripheral

circulation. As mentioned, splanchnic AA affinity is lower, but blood flow to those tissues is between 3 and 4 fold that of mammary glands. Thus, a large proportion of AA not extracted by the mammary glands is recycled to splanchnic tissues, where AA are catalyzed and converted into urea. Therefore, if mammary demand and extraction of AA increased, recycling, catabolism and N excretion to the environment could be decreased.

This study focused, on mechanisms of regulation of protein synthesis that would increase mammary demand for AA and thus, increase post-absorptive efficiency for milk protein. Previous reports indicated that mammary tissue responded to Ile, Leu, Met, and Thr by increasing rates of casein synthesis. Messenger RNA translation rate is regulated by signaling pathways that control initiation and elongation factor activity. In this study, AA did not affect the integrated stress response pathway. However, mTOR and the downstream protein rpS6 phosphorylation were increased and eEF2 phosphorylation was decreased curvilinearly when the four AA were supplied. Translation elongation is inhibited when eEF2 phosphorylation increases. Ile alone affected phosphorylation of the three proteins. Leu reduced eEF2 phosphorylation, but supplying both AA together negated individual effects on eEF2 phosphorylation. Met reduced eEF2 phosphorylation. Thr, increased mTOR and rpS6, and decreased eEF2 phosphorylation. However, Thr negatively interacted with Ile on mTOR and rpS6, and with Leu on eEF2. Despite AA having different effects on signaling proteins, mTOR and rpS6 signals were positively correlated, and these negatively correlated with eEF2.

Isotopically labeled Phe and MALDI-TOF were used to measure incorporation of the Phe into an α-S1 casein peptide with three Phe residues. An innovative approach was described to estimate synthesis rate of labeled and unlabeled casein during the incubation

period. Ile, Leu, Met and Thr increased α-S1 casein fractional synthesis rates (CFSR) when supplied together or individually. The responses were saturable within the range of data studied, and no interaction between AA was detected, indicating additive effects. Surprisingly, AA effects on mTOR phosphorylation and CFSR were not correlated. The CFSR independent responses to individual AA contradict the single-limiting AA theory for milk protein synthesis. That means, if two of these AA have not met requirements, supplementing any of both would cause a response in milk protein synthesis. Moreover, a non-linear CFSR response to each of the 4 AA was observed. In other words, as the supply of AA got close to requirements, the response in milk protein synthesis decreased. Variable responses are not considered by current nutrient requirement systems. Future research should validate these findings at the animal level and determine response surfaces to EAA when dietary CP is reduced (14%). Variable efficiencies and independent AA effects should be incorporated into future requirement systems to adjust requirements and reduce prediction errors. That approach should allow formulation of dairy diets with reduced dietary CP levels and reduced N excretion.